

## Back to Reykjavik (or 1979)

*The new Soviet proposals on European missiles have been welcomed (as they should have been) even though they imply no more than a return to the late 1970s.*

MR Mikhail Gorbachev's proposal, last weekend, that there should be an immediate agreement to get rid of missiles of intermediate range in Europe is unexpected but welcome. For practical purposes, it is the 'zero option' first suggested three years ago by the United States which was one of the ingredients of the package abortively canvassed during last November's breathtaking and abortive summit meeting at Reykjavik. What has changed, since last November, is that Gorbachev is no longer asking that an agreement on intermediate missiles in Europe should wait on agreements on strategic arms and on the ambition of the US president to build a defensive shield against nuclear weapons. It seems a big step forward, although it will be easier to be sure when the negotiators at Geneva have had a chance this week to read the fine print. Meanwhile, there are good reasons why everybody should realize that what is now proposed is a return to the military disposition of nuclear weapons that obtained in Europe in 1979. For Europeans, at least, that was a more comfortable world, but it was in no sense a world free from trouble.

It is also proper, if out of spirit with Gorbachev's evident wish that something should be done to lessen military tensions, to recall that the deployment of US missiles in Western Europe is a direct consequence of the earlier deployment of SS-20 missiles by the Soviet Union. Neither of these developments had been foreseen just a few years earlier, during the negotiation of the SALT II treaty on the regulation of strategic missiles. The unannounced appearance of some hundreds of mobile SS-20s capable of launching nuclear warheads at any target in Western Europe cannot but have engendered a mixture of fright and indignation among governments in Western Europe; by itself, the deployment of the Soviet missiles would have ensured that the SALT II Treaty would not have been ratified by the Senate of the United States (but there were many other impediments to that course in 1979). The practical consequence has been the deployment by the United States of a countervailing force of cruise and Pershing II missiles on the territory of half a dozen West European countries. By agreeing to uncouple consideration of an agreement on intermediate missiles from the other issues on the agenda of the bilateral negotiations at Geneva, Gorbachev has tacitly agreed to roll back these uncomfortable pages of history.

That seems to be the spirit of the chorus of welcome, tinged with idiosyncratic caution, for Gorbachev's proposal. Dr Hans Dietrich Genscher, the Free Democrat foreign minister in the West German coalition government, was characteristically quick to throw his hat in the air in celebration. The statement from Lord Carrington, the secretary-general of the North Atlantic Treaty Organization, that the proposal is a "step forward" will carry great weight, as will that attributed to Mr Richard Perle, the Pentagon's professional sceptic on arms control, that Gorbachev's proposal is "promising". Why are so many people so welcoming? There are some practical reasons; the Netherlands, for example, which is committed to deploy US cruise missiles next year, will be glad if it can be relieved of that politically contentious obligation. Mr Neil Kinnock, the leader of the British Labour Party committed by last October's annual

conference to a policy of unilateral nuclear disarmament may conversely be hoping that an agreement on intermediate missiles will make his policy less of an electoral liability. But the truth is that Gorbachev has timed his proposal well. After the disappointments of the past few weeks, the US president is likely to be more receptive than for months to an agreement such as that now on the table. More than that, after two years of negotiation that seems to have made very little headway, and with the wrangle about the compatibility of the Strategic Defense Initiative and the ABM Treaty threatening to become more bitter, the electorates of the West would like to see some action. So too, no doubt, would Gorbachev's constituents.

That is why a full-throated welcome for the Soviet proposal should be blended with an appreciation of the problems that would survive a deal. The technical objection to both sides' Euromissiles is the ambiguity of their military function. Although not formally classified as strategic, both forces could be used strategically, by being vehicles of threatened attacks on the other sides' cities, for example. At the extreme, they could conceivably (but dangerously) be used to call the supposed bluff of superpowers that have declared their willingness to assist more vulnerable allies by risking their own survival in a nuclear exchange. The potential removal of that risk is an important benefit of Gorbachev's proposal, but other problems of the same kind would remain. One is that both superpowers have scattered Europe with a variety of nuclear weapons designed for use in land battles, all of which could serve to escalate a conflict under way. Nuclear weapons carried by aircraft, of which there are some thousands within striking distance of strategically important European targets, are equivalent to, and as dangerous as, those carried by intermediate missiles. And then there are the British and French nuclear forces, explicitly designed to exert a strategic influence on the course of largely European conflicts. All these ways of making nuclear warfare would survive the acceptance of Gorbachev's proposals.

None of this implies that the game is not worth the candle. Rather, the lesson to be learned is that, in this part of the arms control canvas as elsewhere, no single agreement can be expected to stand on its own for the rest of time. So much is clear from the clutch of apparently hopeful treaties left over from the 1970s, all of which (with the exception of the partial test-ban treaty limiting underground tests of nuclear weapons to the equivalent of 150,000 tonnes of TNT) are being eaten away at the edges by the progress of military technology and undermined by their conceptual isolation. What the erosion of those old treaties shows is that even the most purposeful agreement will not last indefinitely unless it becomes a stepping-stone to other things, most simply another agreement, less tangibly, a better international climate. On this occasion, mercifully, Gorbachev has acknowledged that something will have to be done about battlefield weapons if an agreement can be reached on Euromissiles. What may surprise him is that with his decision not to make such an agreement hostage to agreements on strategic missiles and on ballistic missile defences, he will have improved the chances also of reaching agreements on those still more serious matters. □



# Debtors call the tune

*Brazil's decision not to pay interest on its debts will undermine the stability of the dollar.*

By one of those little ironies, Brazil's announcement two weeks ago that it was suspending interest payments on its overseas debts immediately preceded the meeting of the finance ministers of the six rich countries of the West which declared that the value of the US dollar had declined sufficiently in the previous eighteen months, and that they would henceforth use their influence to keep it more or less where it is at present. In reality the two events are linked, are contradictory and, for what it is worth, full of gloomy portents. This is why.

Brazil owes the rest of the world \$100,000 million in round numbers, much of it dating back to between six and fifteen years ago when the commercial banks of the West were awash with US dollars deposited by oil-producing states neither able nor willing to spend the money on goods. The banks were able to hold the deposits profitably only by lending them to others willing to pay interest on their borrowings; Brazil and similarly industrializing states appeared to be prime candidates. But by the beginning of this decade, the bankers had begun to worry. The sheer volume of international debt had become too large for comfort. Then it emerged that one of the potentially most powerful sources of loans — the economy of the United States — had switched almost overnight from being a lender to a borrower.

The result has been entirely predictable. For the past five years, the reputation of the US economy for flexibility and the size of the federal deficit has kept US interest rates high and the dollar 'strong'. If the propensity of US citizens to save had been greater, the Reagan administration's aversion to tax increases would not have mattered, for the deficit would have been covered (as is the proportionately greater deficit of the government of Japan) by people's willingness to forgo consumption. In the event, people (mostly represented by financial institutions) elsewhere have helped balance the books by buying dollar securities with their savings, accumulating in the process substantial assets, chiefly land, buildings and industrial plant, in the United States. The US trade deficit (\$180,000 million last year) is the counterpart of this process, an arithmetical consequence of the inward flow of capital.

This has been a frightening period for the Brazils of this world, of which there are a great many. High interest rates have increased the cost of debt. Last year, Brazil paid \$12,000 million in interest, roughly 4.5 per cent of its national income. During the same period, the commercial banks of the West have ceased to be the prolific sources of funds they were a decade earlier. Their new-found caution is understandable; as they see it, they would be imprudent to throw good money after bad. The snag is that international institutions such as the International Monetary Fund are incapable of lending on the scale required to help Brazil and the others because their member governments are no longer prepared to equip them for the task, and are in any case not able to raise the funds with which to do so. The decline in the value of the US dollar since the beginning of last year will serve only to exacerbate the problems of the developing industrializing countries, which will find it harder to sell their manufactures on the international markets.

Plainly, this state of affairs cannot continue much longer. The three generic classes which have connived in the past in the creation of these contradictions have an interest somehow to conspire to resolve them. If the banks begin to fall like ninepins because they are forced to acknowledge that the debts that others owe them are worthless assets, there will be an economic crisis. But there are no sanctions that can be applied against sovereign debtors that will not make matters worse. In these circumstances, declarations about the maintenance of the US dollar at some predetermined value cannot carry much weight. It might be different if there were some leeway in the system,

such as might come if the savings ratio in the United States were greater, or if the budget deficit were less (which could not be brought about overnight without precipitating recession). The best solution would be for the still-rich countries of the world to refinance the international lending institutions at a decent level, even at the cost of the measure of domestic inflation this would cause for some of them. Otherwise nature will take its course. □

# What human frontiers?

*The Japanese government has some way to go in the definition of its basic biology programme.*

THE government of Japan has come a long way in the past year in its definition of its Human Frontiers project, first made public in a fog of inexactitude. By a distinctively Japanese process of consultation and iteration, what seemed at first to be a vague programme of research is slowly turning into a programme of basic biological research that could be more than merely worthwhile (see page 8). It is especially intriguing that, on one view at least, Japan's financial contribution to the project would be that of a source of support for research projects arising not only in Japan but elsewhere. It is high time some government broke the mould of the pretence that the pursuit of excellence in research is possible only in its own laboratories. It will be interesting to see what elements of this particular proposal survive in the final prospectus.

It will also be important to see how distinctively Japanese the final plan will prove to be. The grand objective is to design a stimulus for basic biological research by means of a fund estimated at \$250 million a year, rather less than 5 per cent of what is spent each year in the United States by the National Institutes of Health alone. So the question for the planners is not only whether it is possible to define a set of objectives that will give the programme a distinctive sense of purpose but also that of accomplishing something memorable with comparatively modest funds. The question is especially teasing because the government is apparently hoping to use the programme as a means of killing off the legend that Japan lets others concentrate on basic research while bending the energies of its own people towards practical (and profitable) objectives. The diffidence is taken too far (and would be entirely beside the point if there were more support for research in Japanese universities). Meanwhile, with such a relatively moderate budget, the Human Frontiers Project should put diffidence aside and give the international quest for an understanding of life and its aberrations a Japanese flavour.

What might that be like? The obvious temptation is to guess that, because Japanese companies have a splendid reputation in the design and manufacture of instruments, their energies should be harnessed under the rubric of the Human Frontier Science Program to schemes such as that for the sequencing of the human genome, in which instrumentation is bound to play an important part (see Wada, A., *Nature* **325**, 771; 1987). People elsewhere should therefore understand that such a view overlooks the great value of the classical molecular biology by which a growing number of Japanese scientists are distinguished, not to mention the fields such as veterinary medicine in which Japanese scientists have won international acclaim. The Japanese community will be understandably resentful if, in the process of consulting others about new directions, it finds itself typecast as an offshoot of, say, the successful watchmaking industry in Japan. But by the same test, it would be unwise to overlook the opportunity for exploiting the ingenuity of Japan's existing army of skilled designers in the solution of problems in biology which do not normally come their way. Perhaps the best balance between proper resentment of being typecast and the understandable wish to make a mark will be to concentrate the resources of the Human Frontier Science Program on some field now languishing for lack of sufficient ingenuity. Why not ask "How does the brain work?" and see where that leads? □



# AIDS arrives in Soviet Union — (Official)

- Health authorities issue warnings to people
- Scientists seek research links with West

## London

THE Soviet Union has made a U-turn on its attitude to AIDS (acquired immune deficiency syndrome). After several years of castigating it as a disease of decadent capitalism — and, on occasion, attributing its origin to US bacteriological warfare experiments that went wrong — the Soviet media have begun publishing interviews with high-level health officials setting out how to avoid contamination. Immediate measures include the establishment of an anonymous AIDS screening facility at Moscow's No. 2 Clinical Infection Hospital, and, apparently, a decision by the authorities not to invoke legal sanctions against those who seek its assistance. (Homosexual acts carry a sentence of up to five years in prison.)

A mass campaign of 'special publications' on AIDS is planned, and there is talk of a telephone 'hotline' information service. The All-Union Ministry of Health is to establish a network of diagnostic laboratories which will ultimately screen the blood of all donors as well as all patients presenting symptoms indicative of AIDS. A massive training scheme for specialists in diagnostic and treatment of AIDS is also planned. A new specialized clinic for AIDS will be established, and the 40-odd institutes working on AIDS have been promised increased funding.

The turnabout is not entirely unexpected. There have been several hints that Soviet doctors and virologists would be interested in cooperating with the West on AIDS research — "in the interests of science". The new AIDS awareness campaign was first disclosed at the end of last year, but the full-scale information campaign seems to have been held up until sufficient test-packs were available to meet the expected demands. Interviewed by the Moscow *Literaturnay Gazeta* last week, the deputy minister of health, Georgii Khlyabich, castigated Western companies that kept their work on AIDS diagnostics so secret that "we obtained practically no information at all, even though we visited those firms". The Westerners, he implied, were dominated by the profit motive. Soviet scientists, he said, have therefore had to seek their own solution. Intensive research on the "diagnostics and prophylaxis" of AIDS began in 1985, Khlyabich said, and a Soviet diagnostic preparation is now being produced on an industrial scale. Known as IFA-SPID (SPID — pronounced "speed"

— is the Russian acronym for AIDS) the preparation, which was developed by a team headed by Dr Viktor Zhdanov and Dr O. G. Andzhaparidze, is said to be "identical in sensitivity and effectiveness" with Western analogues.

At the same time, a team at the Institute of Immunology of the Academy of Medical Sciences headed by Dr Rem Petrov is working on a synthetic diagnostic agent, Khlyabich said. According to a TASS interview with Petrov, this agent is based on synthetic peptides and known as Peptodskrin. Petrov's team is trying the same approach to develop a vaccine to AIDS.

Until such vaccine is developed, however, (and Khlyabich puts the time scale "at least five years") the main means of AIDS prevention must be what Dr Valentin Pokrovskii of the Central Institute of Epidemiology called "a sober way of life in all its manifestations". Interviewed on Moscow radio, Pokrovskii said that Soviet "social conditions" insure against a major epidemic in the Soviet Union, and urged the advantages of an ordered sexual life. It is those who have had sexual contact with foreigners, he said, who should report for check-up. Mr Gorbachev's campaign against drug addiction and alcoholism, he said, was a major means of AIDS prevention. Pokrovskii said the risk from blood transfusions is negligible, as the Soviet Union buys neither blood nor blood-products, and tests on a "sufficient cohort of [Soviet] donors" has revealed no infection with AIDS.

But last October, Zhdanov said that AIDS was first discovered in the Soviet Union two years before in an 11-year-old girl who before contracted it during a blood transfusion, and, according to the Khlyabich interview, Zhdanov fears an eventual incidence in the Soviet Union of one in 100,000 of the population (say, 2,800 cases).

But whatever the scale of the eventual spread of the disease, the AIDS campaign is presenting considerable logistical problems for the Soviet health service. The endemic lack of drugs and equipment has been a major target of Gorbachev's drive of "open" criticism, and the extra burden of the AIDS campaign will fall on a medical supply industry already far behind target in both quality and quantity. Nor is it only AIDS-related equipment and drugs that will be needed — the entire Soviet medical profession will switch over to the use of disposable syringes. Vera Rich

# Investment by Japan booms in Europe and US

## London

WHILE the European Economic Community (EEC) prepared its anti-dumping case against the Japanese semiconductor industry this week and the US trade authorities once more accused the Japanese of breaching trade agreements, a British report disclosed an embarrassingly high level of inward investment by the Japanese in Europe and the United States.

The European anti-dumping suit, presented to a meeting of the General Agreement on Tariffs and Trade (GATT) at the beginning of this week, cites Japan as exporting microchips to Europe at below their market value. The United States has made similar accusations in recent months but has not confined criticisms of Japanese trading practices to the semiconductor industry. There is growing concern among the US trade authorities that the Pacific basin industries are the primary cause of the US trade deficit.

The figures, highlighted in a report by the Royal Institute of International Affairs, show that more than a third of Japanese overseas investment in the past 35 years has been directed at the United States, accounting for a commitment of \$25,290 million. The figures for Europe are equally stark. In the same period, Japan has poured some \$11,000 million into Europe — more than 13 per cent of Japan's overseas investment.

The size of the investment in both continents is likely to support the view that Japan is using the finance from its trading surpluses to erode indigenous manufacturing and service industries, thereby further weakening the competitive challenge of the United States and Europe.

But the figures also indicate that the investment has proved to be a valuable source of new jobs, again a situation with which indigenous industry has expressed its unease.

According to the study, *Industrial Collaboration with Japan*, the United States is "by a long way" the first choice for Japanese investment. By the end of 1984, there were 370 Japanese manufacturing operations in the United States with much investment being attracted by the overseas marketing efforts of individual states, desperate to create new jobs. By last year, claims the study, more than 20 state governments had individual offices in Tokyo.

Not all have been happy with this approach. Colorado governor Richard Lamm is cited in the report as highly critical of the strategy and the dangers posed to US technology. Bill Johnstone



## Students in India object to AIDS test

New Delhi

THERE is no indication that the Indian government will reverse its decision on compulsory tests for AIDS (acquired immune deficiency syndrome).

The Minister for Human Resource Development, Mr P. V. Narasimha Rao, told the Indian parliament on 25 February that the screening of foreign students for AIDS would continue. Rao ignored suggestions from Members of Parliament that this discriminatory practice could spoil India's relations with African countries. He said AIDS is a serious matter and that the government has decided to deport foreign students who carry the AIDS virus. He also rejected a suggestion that serum samples suspected to be positive for AIDS, on the basis of tests carried out in India, should be sent abroad for confirmation.

Africans, who constitute more than 80 per cent of the estimated 30,000 foreign students, feel the test is directed at them and say it is racist. The foreign student community does not oppose tests on new entrants but is against screening those who have studied in India for several years.

Foreign students of Bombay University failed to turn up for the test scheduled for 16 February. The screening was postponed to 22 February but the International Students Association decided that none of its members would take the test unless local students were also included. Foreign students at Delhi University have also boycotted the test.

Fear, social ostracism and the psychological trauma of being singled out as prospective AIDS carriers have put the educational career of the Africans in jeopardy, their student leaders allege. They say they are shunned by their former Indian friends in the universities, in their apartments and by tradesmen. The Africans fear expulsion from the universities on the basis of unreliable tests showing false positives, or tests conducted by non-experts. They claim that three Kenyan students deported recently from India were found to be AIDS-free in more accurate tests carried out by experts back in Kenya.

The students are agitated because they have been told that failure to take the test would prevent them taking the annual examinations, barely a month away.

Some 1,500 African students who have refused to undergo the test were planning a protest march in New Delhi on 27 February, and it was expected that a large number of sympathizers would attend.

India has so far detected 92 cases of AIDS infection, five of whom died in the past year.

K.S. Jayaraman

## Extra MRC funds earmarked for AIDS research in Britain

London

BRITISH scientists have persuaded the government to increase the budget of the Medical Research Council (MRC) by £14.5 million over three years for research into the prevention and cure of AIDS (acquired immune deficiency syndrome). Most unusually for the MRC, the research is to be centrally directed in an effort to maximize its efficiency.

There will be two programmes of research, one on vaccines and the other on drugs. Each will be organized by a separate director who has yet to be appointed. More of the money is expected to be spent on vaccine research than on drug research, says the MRC's secretary Sir James Gowans, not least because the drug research is more likely to attract industrial sponsorship. £2.5 million will be available for both programmes this year, with £5 million next year and £7.5 million in 1989. Gowans says he has received assurances of longer-term funding.

The two directors will be responsible for commissioning the various strands of their programmes of research. Much of the research will probably be concentrated in three or four large centres with smaller, specific projects being commissioned elsewhere. Both Gowans and Mr Norman Fowler, Secretary of State for Social Services, emphasized that contracts will go to the most appropriate laboratories,

## Compulsory AIDS testing in Bavaria

Hamburg

ANYBODY suspected of being infected by human immunodeficiency virus (HIV), the cause of AIDS (acquired immune deficiency syndrome), will soon be compelled by the Bavarian authorities to undergo a test.

The decision was made by the cabinet (*Ministerrat*) of the Bavarian *Land* government on 25 February. If such people do not appear for testing the health authorities and police will force them to do so.

Male and female prostitutes fall into the risk category, as does anybody seen talking to them. Prisoners and those held on remand are also compelled to take the test. Furthermore, candidates for the civil service will be tested, and anybody whose test proves positive will be rejected.

The other states of the federal republic have criticized the tough stance of Bavaria. The federal minister of health, Rita Süßmuth (Christian Democrat), said that this was the wrong way to deal with the disease. The Social Democrats called the Bavarian initiative "ineffective and hardly acceptable".

Claus P. Dechau

whether they are academic or industrial.

The proposals that attracted these funds were put together by the MRC after consultations with scientists, many of whom would be keen to play a part in the project. Gowans was closely involved in the consultations on vaccine research, while Sir David Phillips, chairman of the Advisory Board for the Research Councils and a leading protein crystallographer, chaired a recent and hurried meeting of molecular biologists with a potential interest in exploring viral proteins that could be targets for drugs against AIDS. Both sides agreed that the two major limiting factors in AIDS research are the lack of sufficient quantities of viral proteins either to be tested as vaccines or to be crystallized for structural studies and the lack of convenient animal models.

Speaking at MRC's press briefing, Professor William Jarrett of the University of Glasgow, said that some of the funds earmarked for vaccines will have to be spent on animal testing facilities. Chimpanzees and, perhaps, gibbons are the only animals that can currently serve as models but the recent report of an HIV-type of virus associated with an AIDS-like syndrome in cats was hopeful. He believed there would soon be immunity trials of subunit vaccines in man. Gowans volunteered himself for such a trial.

At least two laboratories have expressed an interest in filling the need for large quantities of viral proteins. One is the Public Health Laboratory Service Centre for Applied Microbiology and Research, at Porton, which already produces viral antigen for Wellcome's AIDS test. The centre's director, Dr Peter Sutton, has already discussed with MRC how the centre might help. The other is the Natural Environment Research Council's Institute of Virology in Oxford which has expertise in the use of engineered baculoviruses for the production of large quantities of proteins. Its director, Professor David Bishop, is waiting to hear whether the MRC is interested in this project. If not, he has commercial backers in the wings.

Research on the proteins, once available, is likely to proceed in various laboratories. Dr John Skehel's division of virology at the MRC's National Institute of Medical Research is already working on the envelope glycoprotein in conjunction with crystallographer Don Wiley at Harvard. And Professor Tom Blundell's group at Birkbeck college in London is particularly interested in the proteinase of the virus, which has some relationship to proteinases of known structure. One centre for vaccine research seems certain to be Jarrett's laboratory.

Peter Newmark

## AIDS meeting calls for wider testing

Washington

TESTING for AIDS (acquired immune deficiency syndrome) should be offered more aggressively to people in high-risk groups but should not be made compulsory. That is the conclusion of a conference sponsored by the Centers for Disease Control (CDC) in Atlanta last week.

The participants, including state and local public health officials and civil liberties advocates as well as representatives of some high-risk groups, met to discuss the role of serological testing in the prevention and control of AIDS. The CDC will evaluate the recommendations and issue its own report to the government in the next few weeks.

The biggest change that the conference participants advocated was a shift from passive to active testing in treatment centres for sexually transmitted diseases and intravenous drug users. Under this plan, people attending the centres would be tested for AIDS "routinely" unless they specifically objected.

The participants also called for greater confidentiality of test results and protection from discrimination of those tested. Although unauthorized release of test results does not seem to have occurred very frequently, some groups, particularly homosexuals, fear the repercussions of unauthorized release.

Nevertheless Franklyn N. Judson, director of public health in Denver and co-moderator of a panel discussion at Atlanta, stressed the need for keeping confidential records of AIDS virus carriers. He pointed out that for years there have been confidential registries for people with other sexually transmitted diseases, and that it "would be irresponsible not to do this for AIDS". But Judson thinks it unlikely that the US government will issue regulations on confidentiality.

Judson said that cities such as Denver have begun screening blood from heterosexuals who attend the treatment centres for sexually transmitted diseases. So far, all carriers of the AIDS virus have come from an identified high-risk group.

CDC are also conducting a long-term study of blood scheduled to be discarded from hospitals for evidence of AIDS virus antibodies in the hope of compiling data on the prevalence of infection. All patient identification data except for age and sex are removed before blood samples are tested. The hospitals involved are in both low- and high-prevalence areas. By using blood already due to be discarded, the CDC are able to avoid issues of consent.

Steven Dickman

## Special Research Centres to continue in Australia

Sydney

THE Australian government is to continue to fund the Special Research Centres says Senator Susan Ryan, Minister for Education. The centres were previously known as 'Centres of Excellence' at their inception in 1982.

There are at present nine Special Research Centres, which last year received a total of A\$6 million. On the recommendation of a Commonwealth Tertiary Education Commission (CTEC) committee, Ryan has promised that funding for the programme will continue at least at the present level for the three years to 1990. One and a half centres will lose their status as such this year and the rest will suffer cuts of around 10 per cent. The A\$1.5 million saved will go to establish three new centres that will be announced in the federal budget in August. The new centres are likely to be in areas closely related to industry to increase exports of manufactured goods and to answer criticisms that the present distribution is biased towards medicine and biology.

To be cut off this year are the Centre of Policy Studies at Monash University headed by Professor Michael Porter and the group led by Professor R. Bruce Knox working on reproductive biology, which makes up half of the Plant Cell Biology Research Centre at the University of Melbourne, the surviving half being led by Dr Adrienne Clark, the only woman leader among the centres. The axing of Porter's centre was seen by the Conservative opposition as a blatant political move by the Labor government as the centre has been involved in modelling work for the development of tax policies for the opposition and right-wing groups. Ryan maintains that the move was taken purely on the advice of the CTEC committee which

concluded that the published work attributed to the centre of neither the quantity nor the standard required.

The Special Research Centres are opposed by the Federation of Australian University Staff Associations (FAUSA) which considers the centres' budget to be taken from that available for research by the average teaching academic.

The centres were established by the previous Conservative coalition government on the premise that applying concentrated money with guaranteed continuity to the nation's best brains would produce results far in excess of what might be achieved if the money were spent through normal channels. Dr James Beattie, associate professor in chemistry at Sydney University and chairman of the FAUSA Research Committee, points out that the CTEC review was conducted hurriedly with some of the centre assessment panels complaining of lack of time.

According to Beattie, FAUSA would prefer to see the money spent on the general research infrastructure such as buildings, libraries and equipment, at present in a poor state. FAUSA would prefer the funds to be administered by the Australian Research Grants Scheme (ARGS) where the relative excellence of proposed research is subject to broad-based review by academic peers.

Each year, the ARGS distributes about 1,200 grants to a total Australian academic population of about 12,000 scientists. Alternatively, given that the average ARGS grant is worth A\$20,000, the A\$6 million of special research centre money would be enough to fund the work of the 300 researchers whose proposals the ARGS judged to be "excellent" but for which it had no money in 1986.

Charles Morgan

## Boost for Indian high-energy physics

New Delhi

INDIAN physicists are making plans to build a 1-GeV proton synchrotron, to be followed by a 12-GeV Synchrotron, in an attempt to catch up with the West in high-energy physics research. The accelerators will be built at Indore, in Madhya Pradesh, where India is establishing the Centre for Advanced Technology (CAT) at a cost of some \$100 million.

The task of setting up CAT has been given to the Department of Atomic Energy (DAE), which says that the centre will focus on accelerators, lasers and laser technology for thermonuclear fusion. CAT is expected to become a major high-technology centre in the next 20 years.

To start with, DAE hopes to build a synchrotron radiation source consisting of a 25-MeV electron linear accelerator, a booster that will accelerate the electrons to 700 MeV, and two storage rings. The proton synchrotrons are aimed at producing anti-protons and kaons and studying the physics of condensed matter using heavy ions. India at present has a 100-MeV cyclotron in Calcutta, a 5.5-MeV Van der Graaf machine and a 14-MeV pelletron in Bombay.

According to DAE, CAT will be the main research centre for work on laser fusion. CAT will also concentrate on associated fields such as cryogenics and radio frequency systems.

K.S. Jayaraman



# How to lubricate the gears of Texas higher education

## Austin

ALTHOUGH the major centres of higher education in the United States are still on the country's two coasts, Texas universities have been making steady progress towards national prominence. When oil flowed freely, universities in Texas enjoyed the boom. Now, with the state's oil-based economy depressed, higher education is struggling.

When a special session of the legislature last year had to cut \$512 million from the state's \$19,000 million budget, \$200 million came from funds for higher education. But supporters of higher education are preparing to fight as the next round of the budget debate gets under way to restore funds, allowing Texan universities to continue their improvement.

Ammunition for the fight comes from a report released last week by the select committee on higher education. Recognizing that the state's financial difficulties threaten earlier gains in higher education, the state legislature in 1985 created the select committee to consider the problem. After 15 months, including some 30 all-day sessions, the committee's recommendations encompass a variety of issues ranging from faculty salaries to requirements for undergraduate degrees and state support for basic research at universities.

One recommendation that appears to have strong backing is state support for university research. In 1985, the legislature created the Texas Advanced Technology Research Program (TATRP), providing \$35 million for competitive research grants for scientists in the state's public universities. Grants were awarded to five areas, based on a National Academy of Sciences report which identified

promising research areas.

Norman Hackerman, formerly president of the University of Texas at Austin and Rice University and a member of the select committee, says TATRP gave the government of Texas a chance to "get its feet wet" in research support based on



*William Hobby, champion of higher education.* peer review. Hackerman headed the committee's task force on research, which has recommended continuing and expanding the TATRP programme. As well as supporting technology, the report urges the state to award between \$20 and \$40 million — approximately 10 per cent of the amount from the federal government — for basic research at universities. The report also recommends that institutions should receive half of the indirect cost reimbursement accompanying federal grants.

But peer-reviewed distribution of funds presents political problems. The panel chosen to divide TATRP funds would have given 90 per cent of the money to researchers from the University of Texas at Austin and Texas A&M University at College Station. But legislators, jealous of their already high status, prevented them from receiving more than two-thirds of the TATRP funds.

Admiral Bobby Inman, formerly president of the Texas-based Microelectronics and Computer Technology Corporation and originator of the TATRP concept, believes a form of intellectual elitism is ultimately good for the state. In hard economic times, it makes good sense to put resources where they will do the most good, says Inman. If that means just a handful of institutions win grants, so be it.

Resolving regional differences occupied a large portion of the select committee's energy. Economic necessity seemed to require closing some institutions opened during more prosperous times. But closing a university is "harder than moving a cemetery", says Texas commissioner of higher education Kenneth Ashworth.

Many communities appeared before the board to plead their local university's case. One school even rented a hot-air balloon to fly over Austin to protest at its potential demise. In the end, the committee opted to recommend retaining all 37 universities, 49 community colleges, 4 technical institutes and 8 medical and health-related institutions. Instead of closing universities, the committee recommends that some institutions abandon their graduate programmes and stick to undergraduate education. But even that will be opposed.

Ashworth says this is a time for higher education to put the brakes on expansion, and to find ways to guarantee support for existing institutions. He is the first to point out that the cuts in the Texas education budget have had a serious and detrimental impact on faculty recruiting. Texas faculty members earned 10.2 per cent less than the average paid to faculty members in the ten largest states a year ago (Texas is third largest). That figure is expected to reach 14.4 per cent in the current academic year. Unless the new state budget provides more salary funds, Texas universities will have tremendous problems recruiting and retaining staff, says Ashworth.

Hans Mark, chancellor of the University of Texas system, agrees with Ashworth that raising salaries is critically important to maintain the health of higher education. But he "emphatically disagrees" with Ashworth's suggestion that the time is not right for expansion. Mark is actively seeking both federal and private funds to help establish new facilities and attract top talent. Mark believes there is reason for optimism about state revenues. He suggests that if oil prices reach \$20 per barrel, and certain short-term increases in sales tax are extended, possible budget shortfalls will not materialize.

A powerful champion of higher education in Texas is Lieutenant Governor William Hobby. A member of the select committee, Hobby has been lieutenant governor since 1972 and is well versed in the intricacies of Texas politics. Hobby talks with pride of the state's academic achievements but bemoans the fact that Texas has less than one-tenth the number of members of the National Academy of Science that California has. Hobby has worked hard to persuade the state's congress that a strong educational system is needed to restore Texas to economic health.

But there is a danger that selling education for its economic benefits may backfire if the economy does not improve. Larry Temple, an Austin lawyer who chaired the select committee, says it is hard to get politicians to take a 20 or 30 year perspective on the benefits of education. But he likes to remind people that, no matter what size the Texan oil reserves may be, it will take educated minds to find them.

Joseph Palca

## New Wellcome unit

### London

TAKING £1 million from its swollen coffers, the Wellcome Trust has set up a new unit within the University of Glasgow. The Wellcome Unit of Molecular Parasitology, directed by Dr Andrew Tait, is to concentrate on molecular and genetic investigations of the basic biology of parasites.

The unit, which is guaranteed for five years, is the first to be funded by Wellcome Trust since it announced that it would be looking for suitable long-term university projects to fund with the extra income derived from its sale of 25 per cent of its (until then wholly-owned) shares in Wellcome plc, the pharmaceutical group whose share have doubled in value in the last month largely because of the anticipated sales of Retrovir (AZT) as a treatment for AIDS.

Peter Newmark



# SDI gives NBS a free-electron laser for basic and applied research

Washington

RESEARCHERS using high-intensity radiation sources will soon have an important new research tool. The National Bureau of Standards (NBS) is building a Free-Electron Laser (FEL), due to be completed in 1990, at its laboratories in Gaithersburg, Maryland. Unlike other FELs constructed so far, this will operate at wavelengths in the near-ultraviolet and visible regions as well as in the infrared, and at a much higher average power. It will be used in fields ranging from atomic physics to nuclear medicine.

The \$4.9 million cost of the FEL will be borne by the Strategic Defense Initiative Organization through a contract administered by the Office of Naval Research and the Air Force Office of Scientific Research. The FEL will give its designers familiarity with the operation of free electron lasers. This knowledge could then be applied to the construction of lasers operating at even shorter wavelengths, perhaps for military purposes.

The new laser was conceived by FEL theorists at the Naval Research Laboratory as an adjunct to the 185 MeV continuous-wave 'racetrack' electron

several points along their path through the accelerator. The full 185 MeV of energy, corresponding to the shortest wavelength, will be achieved only when electrons pass through the entire microtron. Adjustments to the mirrors in the laser itself will be required to provide this broad range, but project director Samuel Penner considers this problem easily surmountable.

The fact that the FEL will be tunable over such a broad range of wavelengths may make it particularly suitable for medical research. For example, researchers at Harvard Medical School are using lasers in experiments on animals to break up cholesterol deposits in arteries. These experiments depend on fine-tuning the wavelength so that the laser beam

destroys the blockage without hurting the artery wall. (The FEL itself will not be useful for surgery until fibre optics are developed that can carry enough power.) In addition, the Food and Drug Administration is interested in using the FEL to test the efficacy and safety of the lasers now used in medicine.

Penner says that the fact that light pulses from the FEL will be synchronized with the pulses of synchrotron radiation from the microtron means that the FEL will be especially useful for 'pump-probe' experiments in which a pulse of light is used to excite an atom or molecule to a higher energy, and a second pulse to analyse the effects of the first. The high power provided by the laser will allow the FEL to 'read' these excitations even if it is not tuned to exactly the right wavelength. According to Penner, the NBS laser will be the first FEL to be used for such a variety of tasks.

Steven Dickman

## Planetary scientists appeal for 1990 launch of Mars Observer

Washington

THE US Planetary Society, headed by chief spokesman and standard-bearer Carl Sagan, rallied support on Capitol Hill two weeks ago for the Mars Observer, a NASA (National Aeronautics and Space Administration) planetary mission left temporarily grounded in the aftermath of the Challenger accident. In evidence to the Senate Subcommittee on Science, Technology, and Space, Sagan stressed the importance of the Mars Observer as a step towards (ultimately manned) exploration of Mars, an objective he suggested would revitalize sagging NASA morale.

The Mars Observer, now set for a 1992 launch, was originally to leave Earth in 1990 to spend a martian year (roughly 687 Earth days) in a low Sun-synchronous orbit monitoring the magnetic fields, atmospheric circulation, gravity fields and chemical composition of Mars by remote sensing. It would also gather data on seasonal fluctuations in the parameters determining geochemical cycles and would provide clues to what happened in the planet's climatological history to dry up the once-extensive water supplies.

But the shuttle timetable until 1990 has only three slots for planetary missions, now filled by the Galileo mission to Jupiter, the Magellan radar mapper mission to Venus and the Ulysses mission to explore the polar regions of the Sun. Ulysses, a joint project with the European Space Agency (ESA), has already been delayed several times. Further delay could be acutely embarrassing to NASA.

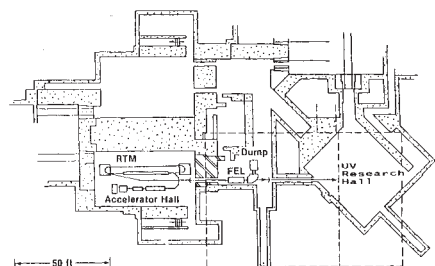
The decision to postpone the Mars Observer launch until 1992 provoked and outcries from the planetary science community

and a letter-writing campaign, sponsored by the Planetary Society, which resulted in over 20,000 letters to congressmen and NASA officials.

Delaying the launch may put the United States behind the Soviet Union in the exploration of Mars. The Soviets have recently brought forward from 1994 to 1992 plans to deploy a balloon probe to the surface of Mars, coinciding with the proposed launch date for Mars Observer. Next year, the Soviet Union will also launch a remote sensing mission of the Martian moon Phobos; former NASA Jet Propulsion Laboratory director Bruce Murray says their plan is "extremely elegant".

Sagan, Murray and Congressman George E. Brown Jr. (Democrat, California) advocate a joint programme of Mars exploration with the Soviet Union. Sagan says a national commitment to put a man on Mars would have the same effect as the Apollo lunar programme, providing a unifying goal to put NASA back on track.

The shuttle is still due to launch Mars Observer, although expendable launch vehicles, such as the Air Force Titan 34D, are acceptable alternatives. But Congressman Edward P. Boland (Democrat, Massachusetts), chairman of the House of Representatives appropriations subcommittee that oversees NASA, objects to the Mars Observer using an expendable rocket. He claims the extra \$100 million needed to launch Mars Observer in 1990 using a relatively outdated Titan 34D is a poor investment, considering the impending development of a more advanced Titan IV rocket schedules for 1991. But waiting until 1992 will add an additional \$90–100 million to the price. Carol Ezzell



Plans of the free-electron laser and racetrack microtron.

accelerator already under construction at NBS. The accelerator acts as an energy source for the laser by providing regular pulses of electrons which, by interaction with carefully shaped irregular magnetic fields, give off energy as radiation.

According to NBS physicist Xavier Muruyama, people are "throwing away a lot of available light" with the lasers now in use. The continuous-wave accelerator, or 'microtron', will allow the new FEL to operate over a much broader spectrum of wavelengths than other free-electron lasers. Its range will extend from the 10  $\mu\text{m}$  infrared region to the ultraviolet region at 0.2  $\mu\text{m}$ . Furthermore, because the repetition rate of the electron pulses feeding the laser will be essentially the 100 MHz frequency of the microtron, the average power output of the FEL will reach hundreds or even thousands of watts.

The remarkable range of wavelengths is due both to the microtron's power and to its design. Electrons can be deflected at

# Japanese Frontiers programme fillip to research

## Japan

THE Human Frontier Science Program, Japan's answer to Star Wars and Eureka, could be the best thing that has ever happened to bioscience research in Japan. Much will depend on the next few weeks and months as the ministries involved debate the recommendations of foreign and Japanese scientists and try to secure financial support from the Ministry of Finance, Japanese industry and foreign governments.

The Human Frontier Program, as it used to be called, first emerged from the Ministry of International Trade and Industry (MITI) about a year ago (see *Nature* 320, 296; 1986) alongside a similar proposal, the Human and Earth Science Program, put forward by the Science and Technology Agency (STA). Both advocated international research in basic bioscience for the 'benefit of all mankind' and agreed with the Maekawa report, released shortly after, which recommended that Japan should contribute to new science and technology in the 21st century by promoting international collaboration in basic research.

Frontiers is aimed at defusing Western criticism that Japan is only a consumer of the fruits of basic research. There was a hint that the Prime Minister, Mr Yasuhiro Nakasone, would propose the programme at the Tokyo summit in May last year. But competition between the STA and MITI, the vagueness of the Frontiers programme, and a busy summit schedule led to postponement.

Nakasone, who has long been a backer of the life sciences (see *Nature* 240, 185; 1972), continues to push the programme. Last year, after behind-the-scenes negotiations between seven ministries, Frontiers re-emerged as the Human Frontier Science Program, a compromise, at least in title, between the MITI and STA proposals.

In mid-December, a feasibility study committee and two working groups of top Japanese scientists were created to develop the programme under the leadership of Michio Okamoto, former president of Kyoto University and chairman of the Committee on Policy Matters of the Council for Science and Technology (headed by the prime minister). Several meetings of both the committee and working groups have been held, including two on 29 January and 12 February at which foreign scientists from the summit nations and the European Economic Community were invited to give their views on how the Japanese government programme should be run.

A 94-page report compiled by MITI's Agency of Industrial Science and Technology in November last year formed the

basis of discussion. This report proposes the creation of all the necessary components of an android (artificial brain, eyes, organs, muscles and tissue) through the study of functions of the human body. These 'genuine intelligent robots' might help solve the 'software crisis', Japan's inability to produce good software, and help understand 'technostress', an ailment brought on by working at the man-machine interface.

Most of the report's proposals are less ambitious although on a large scale. The first chapter covers the study of biological functions at the molecular and cell level.

The second chapter deals with functions of the whole organism such as hearing, sight, smell, touch, motor function and thinking and the third with the technology to carry out the research, including the development of an X-ray microscope, a 30T NMR, and ion- and electron beam-probes.

But the report represents only MITI's views. Okamoto and several other scientific advisers favour a programme that would place more emphasis on pure research. In contrast, MITI believes that research should have commercial applications and that pure research will not win funding from the Finance Ministry. The problem is deep-rooted. Engineering has dominated Japan's university system since its establishment in the last century, pure science taking a peripheral role, and, for most Japanese, research means applied research.

Among other possible projects now under consideration is sequencing of the whole genome, one of the original proposals for the STA's Human and Earth Science Program. The STA, in collaboration with Seiko, has been developing an automatic DNA sequence analyser, and the leader of this project, who chairs one of the Frontiers working groups, has long advocated automated processing of DNA sequences such as the human genome. Genome sequencing would fit in with the proposed 20-year timescale of Frontiers and there have already been calls from the United States for collaboration with Japan and Europe on this huge project. But there are fears that megasequencing would absorb too large a proportion of the Frontiers budget. MITI is hoping for ¥40,000 million (\$270 million) a year (although the budget may be less in the beginning). Most of this would have to come from the government, but industry is expected to contribute. Nakasone will almost certainly propose the Frontiers programme at the next summit in Venice in June, and Katsuhiko Umehara, deputy director of MITI's feasibility study, believes that some other countries will contribute funds.

Sir Walter Bodmer, director of research at the Imperial Cancer Research Fund, said at the meeting on 12 February that he thinks "it is most unlikely that within the United Kingdom we would find a large amount of extra money, if any extra money, to support such a program". But there is "enormous scope for collaboration" within the framework of existing activities and institutions, "if the programme is of a high quality and attractive". Other overseas participants concurred.

Both Bodmer and Professor Joshua Lederberg, president of Rockefeller University, urged Japan to include research directed at the understanding of human diseases, such as parasitic, viral and genetic diseases.

According to a tentative plan outlined at the 12 February meeting, Frontiers will take the form of a granting agency (like the Rockefeller Foundation) which will distribute research grants to international research teams; researchers and information will be exchanged through an international fellowship programme for young researchers and international workshops (like the Gordon Conference) and domestic (Japanese) basic research would be considerably enriched and reinforced in line with the programme.

If the programme is implemented and Frontiers supports basic research, it will be a major turnaround in Japanese science policy. Until now, the Finance Ministry has been frugal on funding basic research. But with the backing of two powerful ministries (MITI and STA) and the prime minister, funding of the magnitude envisaged may well be possible.

One hundred and fifty million yen (\$1 million) has been allotted to the feasibility study in fiscal year 1987 on top of the ¥20 million for this fiscal year, a clear indication that the government is committed to Frontiers. And Japan will invite ten eminent scientists from around the world to discuss the programme in a "wise men's conference" at the Royal Society in London on 1 April.

The other five ministries represented at the Frontiers meetings (the Foreign Ministry, the Ministry of Education, Science and Culture, the Health and Welfare Ministry, the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Post and Telecommunications) officially maintain a neutral stance on the programme. But the successful launching of Frontiers at the summit would be in the interests of the Foreign Ministry. The Education Ministry, on the other hand, is worried that Frontiers could cut into its science budget, but that would be counter to the spirit of the programme and the Maekawa report's recommendation to promote basic research. **David Swinbanks**



## Commercialization of British radio given an amber light

London

UP to three national commercial radio networks and hundreds of community radio stations will emerge from the UK government's tacit approval dramatically to change the rules that constrain the number of UK radio stations.

The new guidelines, published in the form of a green paper (consultation document) last week entitled *Radio: Choices and Opportunities* (Cmnd 92, HMSO, £5.00), will end the monopoly of local commercial stations. Most of these stations enjoy sole commercial broadcasting rights in their areas and may now be challenged by community radio and national networks.

New broadcasting legislation will need to be introduced in the next session of parliament to allow the change. The British Broadcasting Corporation (BBC) will lose two of its seven frequencies. It will be given one in return in 1990 when more frequencies become available. International agreements will provide additional VHF/FM frequencies for broadcasting between the end of the decade and 1995. The government says these will provide "scope for new national and local services".

The national commercial radio service could fuel controversy. The advertising revenue on which the local stations depend could be threatened.

But the national network may have a higher proportion of speech to music and

should be more of a challenger to the BBC's news channel, Radio 4, than the local commercial stations. That at least is the idea of the Independent Broadcasting Authority (IBA) currently the watchdog



Douglas Hurd — planning a radio revolution

of the commercial radio stations.

The government does not want to restrict any commercial venture and is unlikely to impose a formula like that suggested by the IBA. At the community level there is expected to be an explosion.

Mr. Douglas Hurd, the Home Secretary says that "The values and traditions established by our history of high-quality public service broadcasting should not be lost, there can soon be a more varied pattern of radio services and more choice for the listener".

Bill Johnstone

## US Congress debating aid for Pakistan

Washington

SPECULATION that Pakistan has "crossed the nuclear weapons threshold" put a crimp in last week's congressional hearings about a \$4,020 million US aid package to Pakistan. The Reagan administration wants the six-year package, an increase of \$800 million over the last allotment, for economic and military support of Pakistan in the face of the continued Soviet presence in Afghanistan. But Congress is expected to ask some tough questions about Pakistan's nuclear capability.

Since 1985, US aid has been contingent on the US president verifying to Congress each October that Pakistan does not have a nuclear device. A report released this week by the Carnegie Endowment for International Peace indicates that Pakistan either "possesses all of the components needed" to manufacture an atom bomb or else "remains just short of this goal" because of a lack of enriched uranium.

The report, written by Leonard Spector, recounts the history of Pakistani attempts to acquire nuclear weapons technology.

Spector cites as one example the purchase of a plutonium reprocessing facility from France in the mid-1970s which would have permitted Pakistan to accumulate nuclear weapons material. The United States cut off aid to Pakistan in 1976 over precisely this issue. Deliveries for the facility were ultimately suspended and US aid reinstated in 1977. Aid was cut off again in 1979, when it had been found that Pakistan had been acquiring technology for uranium enrichment, and was restored only in 1981, after Soviet forces appeared in Afghanistan.

The administration recently warned Pakistan that US aid may be cut off once again if Pakistan is found to possess even the makings of a bomb. In a speech in Islamabad on 16 February, US Ambassador Deane Hinton warned that under such conditions, it would be "open to question" whether the president could make the necessary certification. Pakistan continues to deny that it is developing nuclear weapons. Congress will decide on aid in the next few months.

Steven Dickman

## Government and students make peace in Spain

Barcelona

THE Spanish minister of education and science, José Maria Maravall, has signed agreements with most of the student organizations that led demonstrations against the Spanish educational system during the past two months. Some radical student organizations have decided to continue the movement, but most students have returned to their classrooms. The agreement is based on a proposal by the minister that includes a gratuity for secondary school students under 16 and for university students from families under a certain income, a substantial increase in the number of fellowships, reforms in the secondary schools and a revision of the entry requirements for universities.

Both sides claim victory. The students say they have forced the minister to negotiate and to accept some of their points. He in turn has declared that the agree-



ment does not mean a departure from his policy but only an acceleration of the reforms already under way.

Most students have returned to class but the unrest has spread to previously unaffected universities. At the Autonomous University of Barcelona police entered the campus to expel students occupying the main administrative building, and the university was closed for two days. This was the first such incident in Spain for more than ten years.

It is clear that the agreement between the minister of education and students will be costly. It is calculated at about 40,000 million pesetas per year (£200 million), almost twice the budget of the CSIC (the Spanish research council). It is not clear where the money will come from and some people fear that proposed increases in university or research funds or salaries may be frozen as a consequence.

Pedro Puidoménech

## Evolution and optimization

SIR—Joe Felsenstein opens his review<sup>1</sup> of Sewall Wright's biography by W.B. Provine<sup>2</sup> in these words: "Evolutionary theory in the 1960s and 1970s was dominated by optimization, by the search for the quantity evolution would maximize. The mean fitness of a population was not precisely what natural selection would maximize in a Mendelian genetic system, so perhaps if we did enough theory we could find out what was. The picture hanging on our wall was of R.A. Fisher, who had emphasized the importance of natural selection in large populations."

This might be taken to imply that Fisher engaged in the search to which Felsenstein refers, as many seem to think. This is not the case; indeed, his students from the late 1950s (I write as one of them) were taught *not* to think in such terms, and we developed a marked scepticism towards those many who did. As early as 1941, Fisher<sup>3</sup> railed against any such optimization: "Wright's conception ... that selective intensities are derivable, like forces in a conservative system, from a simple potential function dependent on the gene ratios of the species as a whole, has led him to extensive but untenable speculation."

To this evidence can now be added the recently published letter<sup>4</sup> that Fisher wrote to M. Kimura on 3 May 1956: "In considering the original statement of what I ventured to call 'the fundamental theorem of natural selection', I had, of course, considered the relation between such a situation and that in which a potential function existed, for my mathematical education lay in the field of mathematical physics."

As you realize, I preferred to develop the theory without this assumption, ... Of course I realise that Sewall Wright has often argued as though such a potential function must exist, ..."

In the matter of optimization, the picture on the wall was that of Sewall Wright, and it carried the caption "The inventor of the notion of the *adaptive topography*, the *adaptive surface*, and the *fitness function*".

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## Foreigners in Japan

SIR—As a British scientist temporarily in Japan participating in a programme of collaborative research sponsored by the Japanese Foundation for the Promotion of Cancer Research, I should like to add a comment to David Swinbanks' article "British are reluctant travellers" (*Nature* **323**, 571; 1986).

It is not difficult to understand, particularly when viewing the situation from the United Kingdom, the apprehension about living and working in Japan that may be in the minds of British scientists. However, once in Japan many of the difficulties one expected to face are, in reality, much less serious than they appear from the United Kingdom. In fact, many of the so-called 'problems' are a source of real pleasure in the interest and the very challenge which they present.

The 'challenge' of Japan should be viewed in a positive way because there are so many things to be gained by spending time here. First, it should be recognized that many of the research centres and university departments in Japan are of a very high standard and have truly earned their international reputation for excellence. One can therefore realistically expect the time spent here to be scientifically productive and successful. Of course, one is expected to work hard and the normal working hours are longer than those in the United Kingdom, but this should not be a detraction. In most research groups this is

hardly noticeable as there is a strong feeling of companionship and very much a community spirit.

Second, although one might be apprehensive about the language problem and differences in culture, the Japanese are a very generous and helpful people. My colleagues here go out of their way to ensure that, as far as possible, my every need is met, and that the communication problem both inside and outside the laboratory does not lower the quality of my life here.

The concern about finding employment back home is understandable but should not be allowed to over-influence a decision to come to Japan. At a time when efforts are being made by both industry and government to increase British interactions with Japan, experience in Japanese science and Japanese culture must be an advantage. For those who wish to return to an industrial position, I suggest that they make contact with companies before departing for Japan. Certainly, the one-year fellowships that the Royal Society plans to offer to those returning to the United Kingdom should cushion the problem of finding an immediate position.

For those who are offered the opportunity of participating in research in Japan, to fail to accept it is to miss the chance of a lifetime.

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## Science in Iberia

SIR—Your report on tropical research in Portugal states that "in the first major outbreak [of coffee leaf rust], towards the end of the nineteenth century, Ceylon's coffee industry was utterly destroyed" (*Nature* **324**, 331; 1986). To be precise, British entrepreneurs began to develop coffee production in Ceylon in 1803, increasing annual production to over one million pounds within 50 years. But the first major outbreak of coffee leaf rust caused by *Hemileia vastatrix* occurred in 1869, and not "towards the end of the nineteenth century"<sup>1</sup>. As it was one of the classic cases in the history of phytogeography of destruction of plantation monoculture by a pathogen, the record should be put straight. Ceylon has been a tea producer since the 1870s.

In your coverage of science in Spain, you record the activities of two Spanish Nobel laureates, Ramon y Cajal (1906) and Severo Ochoa (1959). It would be fair also to mention Portugal's 1949 Nobel laureate in sciences, Antonio Egas Moniz (1874-1955) who is recognized for his pioneering work in prefrontal lobotomy and in cerebral angiography; a junior contemporary of Ramon y Cajal, the Portuguese neurosurgeon was also politically active. He served as a deputy in the Portuguese parliament from 1903 to 1917, when he was named as ambassador to Spain and was also appointed as the Minister of Foreign Affairs. Moniz also served as the president of the Portuguese delegation at the 1918 Paris Peace Conference, before retiring from politics in the following year<sup>2</sup>.

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## Iberia reversed

SIR—Perhaps it is laboursing the obvious, but it seems that the cover of *Nature* **324**, No. 6095, which depicts a Catalonian view of Iberia in 1375, was placed upside down if one subscribes to the convention that north is up. Inversion of the cover might allow more readers to appreciate correlations with modern Iberia.

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Many early maps were turned round in order to make the best use of the paper without altering the format of the completed atlas. — Editor, *Nature*.



# Supernova almost on the doorstep

*Last week's discovery of a supernova outburst in the Larger Magellanic Cloud is an important opportunity for astronomers of several kinds, but one chance has been missed already.*

ASTRONOMERS have been handed a potential treasure trove with the discovery last week of a supernova 155,000 light years away in the nearby satellite galaxy, the Larger Magellanic Cloud (LMC). The exploding star, 10,000 times brighter than any other object in the LMC, provides not only an unprecedented opportunity to study the development of a supernova, but also a beacon by which interesting constituents of the intervening matter can be studied. Perhaps most significantly of all, neutrinos emitted by the initial explosion are thought to have been detected. Not surprisingly, every observatory within sight of the object (predominantly in the Southern Hemisphere) and every appropriate satellite has been rescheduled to monitor the event.

Three astronomers share the credit for the discovery on 24 February. Ian Shelton, of the University of Toronto, found the very bright object by good luck on a three-hour exposure taken at the Las Campanas Observatory in Chile. Oscar Duhalde, a night assistant at the same observatory, spotted the source with the naked eye, as did Albert Jones, an amateur observer of variable stars living in New Zealand.

It turns out that this patch of sky had been photographed by other observers over preceding nights, from which it seems that the supernova was first readily observable (at magnitude 6) on 23 February. Since then, it has brightened by about two magnitudes and is expected to reach its peak brightness within a few days. Meanwhile, the object has been detected at ultraviolet, radio and, very tentatively, X-ray wavelengths and has been granted the inspiring title 1987a.

The neutrino detection was announced by C. Castagnoli and other members of an Italian/Soviet collaboration working at the Mont Blanc neutrino observatory. Five neutrino pulses of energies above 7 MeV were observed at 0300 Universal Time on 23 February — nine hours before the first optical detection.

Happily, and for the first time, it has also been possible to pinpoint the star whose demise has so brightened the lives of astronomers. The object appears to be the blue B-type supergiant Senduleak -69 202 with a mass between 30 and 50 times that of the Sun. This identification agrees well with the notion that, at the end of their nuclear-burning lifetimes, stars of more than a few solar masses undergo a

catastrophic transition, shedding much if not all of themselves in the form of a rapidly expanding shell of gas and often, if not always, leaving behind a compact remnant such as a neutron star. Although there are many such stars in our Galaxy, their explosive ends, reckoned to occur on the average once every 50 years, are obscured — like many of the interesting features of the Galaxy — by gas and dust. Thus astronomers have generally had to make do with such explosions detected in other galaxies.

The new supernova is sufficiently close to offer the prospect of resolving the expanding shell within only weeks of the outburst, although the maximum angle of ten milliseconds of arc that will be subtended by the bright photosphere — the deepest part of the shell visible — comes tantalizingly close to the limits even of speckle interferometry. According to Paul Murdin, of the Royal Greenwich Observatory (RGO) in Sussex, spectral lines of hydrogen and helium have been detected from the shell, which is expanding at 16,000 km s<sup>-1</sup>, but the wide spread of relative gas velocities is for the time being smearing out most of the interesting spectral features beyond recognition. This is no doubt particularly frustrating for those studying a phenomenon that makes supernovae unique and crucial for an understanding of the chemical evolution of the Galaxy — the ejection of heavy elements into the interstellar medium, including not only the residues of the star's nuclear cycle but also products such as short-lived isotopes of nickel and cobalt and stable heavy elements synthesized only during such explosions. Clearly, the neutrino detection is particularly exciting in this context.

Meanwhile, intriguingly, nobody has yet convincingly shown theoretically that supernovae should occur at all. Everybody seems happy that the inert iron core left at the end of a star's nuclear life should, if the star is sufficiently massive, undergo a phase during which electrons are captured by nuclei, thereby removing the capacity of the core to resist the weight of the overlying stellar material and producing a rapid implosion. The core soon reaches nuclear densities and the implosion stops, but nobody has been able quantitatively to explain what happens next. The hand-waving idea is that a shock-wave propagates outwards and expels the outer layers. Specifically, according to Martin Rees of the Institute of

Astronomy in Cambridge, neutrinos are released which should be the agents of such disruption, but their mean free paths are too long to be modelled as 'simple' shocks. Some calculations end with a black hole into which the entire star collapses, while others predict a neutron star remnant with outer gaseous layers whose behaviour cannot be well predicted.

What all this means in practice is that everyone is going to want to see what 1987a leaves behind. A hot neutron star would be detectable by its emitted X-rays, although for the first few months these would be obscured by emissions from the scattered energetic particles within the shell. Moreover, according to X-ray astronomers working with the Astro-C/Ginga satellite, observations will be greatly complicated by the nearby bright X-ray source LMC X-1.

The dynamics as well as the nature of the object left behind will absorb much attention — if a neutron star, will it have a high transverse velocity and will it rotate so rapidly as to act as a pulsar?

As if all this were not compelling enough, the supernova also provides an opportunity to explore the dynamics and the history of the material lying along the line of sight. Already, according to Max Pettini of the RGO, absorption lines of calcium, sodium and magnesium have revealed at least twenty distinct gas motions, many within the LMC and others within our own Galaxy. But the brightness of the object should also allow the detection of comparatively weak absorption features due to trace isotopes whose abundances constrain theories of primordial nucleosynthesis (such as <sup>7</sup>Li) or galactic chemical evolution (such as <sup>13</sup>C/<sup>12</sup>C). Previous studies of this kind have been confined to the region within 300 light years or so of the Sun, but now there seems to be a prospect of similar observations within the LMC. The point is especially intriguing because the LMC is thought to be chemically much younger than our Galaxy.

So most astronomers are understandably delighted, but a small sub-group of them, doubtless now impotently furious, has already missed its only chance to exploit this event. The most aspherical type of core implosion to produce 1987a would also have generated gravitational waves. Maddeningly, no cryogenic detectors were operating but two bar detectors at the University of Maryland were taking data yet to be examined. **Philip Campbell**

## Radioastronomy

## A new signpost in the sky

Ray P. Norris

SINCE the discovery in 1951 of radio emission from neutral hydrogen in our Galaxy, radioastronomers have persistently been seeking other molecules, and additional transitions of detected molecules, with which to probe the interstellar medium. Recent rapid advances in receiver technology have prompted an ever-increasing rate of discoveries of new transitions, so that nowadays the detection of a new transition rarely attracts much attention. The discovery of a new methanol maser, reported by W. Batrla, H.E. Matthews, K.M. Menten and C.M. Walmsley on page 49 of this issue, is surely an exception to this trend of complacency. Batrla *et al.* have discovered not just another weak transition of a well-known molecule, but a source of strong maser action whose intensity rivals that of the well-known OH and water-vapour masers (Elitzur, *M. Rev. Mod. Phys.* **54**, 1225–1229; 1982). The significance of their result lies not so much in its immediate astrophysical implications, but in its implications for the observational study of star formation.

OH and water-vapour masers have been studied extensively since their discovery in the late 1960s in regions of star formation and around old, dying stars. In only a few cases, such as the OH emission from OH infrared stars, do we have any understanding of the inversion mechanisms that must be present to cause the maser action. Despite this staggering ignorance, these masers have turned out to be powerful tools for radioastronomers, primarily because of their sensitivity to physical conditions in the surrounding gas. Observations of masers can, in principle, reveal something of the molecular abundance, density, velocity, temperature and magnetic field. In addition, their high intensity and small size renders them easily accessible to radio telescopes (Norris, R.P., Diamond, P.J. & Booth, R.S. *Nature* **299**, 131–134; 1982). Thus, OH and water-vapour masers stand as signposts in the swirling interstellar gas cloud, indicators of the conditions and kinematics of the gas around very young and very old stars. It is fortunate that the OH and water-vapour masers occur in different regions of the gas, allowing two different regions to be probed.

Batrla *et al.* now add a third type of signpost. Unlike the other, weaker maser transitions of methanol and formaldehyde, the new methanol masers are strong enough to be studied in fine detail by radio synthesis telescopes, and their spectra show structure that promise the complex detail familiar from OH and

water-vapour observations. Because the methanol masers probably occupy a different regime from the OH and water-vapour masers, they offer a chance to add pieces to some very sparsely filled jigsaws.

The sources in which the methanol masers were discovered are the old faithfuls of the OH and water-vapour literature. Each is a known region of active star formation in which OH and water-vapour masers have already been found. The search will now be on for methanol masers in other types of object. Extragalactic methanol masers will no doubt soon feature in these pages, and diverse types of object, ranging from comets to supernova remnants, will be fair game for maser-hunters.

These methanol masers suffer, however, from one major drawback. They have

been discovered too late for the radio-astronomy lobby to try to safeguard (as was done for hydrogen atoms and OH molecules) that portion of the radio-frequency spectrum in which they lie. Symptomatically, Batrla *et al.* were unable to study several sources because of satellite interference. By the same token, few radioastronomy telescopes will be equipped to study the frequency of this methanol transition (12.179 GHz), even though it is technically very accessible. One hope lies in the common availability for this band of commercial satellite receivers, which could easily and cheaply be fitted to existing radioastronomy antennas. Whether this will actually happen will depend on the degree of pressure which interstellar astronomers are able or willing to apply to their engineering and administrative colleagues, and the extent to which such observations would be sabotaged by satellite interference. □

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## Protein evolution

## Positively darwinian molecules?

Andrew Leigh Brown

ACCORDING to the neutral theory of molecular evolution, almost all nucleotide substitutions that are fixed during evolution are selectively neutral, or effectively so. That is, the absolute value of  $s$ , the selection coefficient, is much less than  $1/2N_e$ , where  $N_e$  is the effective population size<sup>1</sup>. It is now well known that synonymous nucleotide substitutions, which do not lead to an amino-acid change, occur at least 2–3 times more rapidly than those substitutions that do cause such a change, during the evolution of most proteins. In addition, in pseudogenes (defective coding sequences sometimes found in multigene families) the rate of non-synonymous substitution accelerates until it equals the synonymous rate. Thus, at the molecular level, rapid evolution has become associated with the lack of any functional difference between substituted alleles. But exceptions to such generalizations can often be more interesting than the rule. Two recent studies<sup>2,3</sup>, one reported by R.E. Hill and N.D. Hastie on page 96 of this issue<sup>2</sup>, provide just such an exceptional case.

Serine proteinase inhibitors are a large group of proteins found in many prokaryotes and eukaryotes. Several families have apparently evolved the inhibitory properties independently, and the interaction of these inhibitors with their target proteinases has been studied in considerable detail (see ref. 4 for a review). A region sometimes called the reactive

centre, which may be duplicated within the molecule, interacts with the active site of the enzyme. One peptide bond within this region is the target for cleavage (see figure). Cleavage occurs extremely slowly, however, and the cleaved products also form a very stable complex with the enzyme. The result is a series of very effective inhibitor molecules. The biochemistry of inhibition is fairly well known for all such inhibitors and so is the precise physiological role of some: for example, controlling the multiple plasma proteinases of proteolytic cascades such as blood clotting. But there are many serine proteinase inhibitors whose physiological role is unknown.

Among the serine proteinase inhibitors of mammalian blood plasma<sup>2</sup> and of ovomucoids (serine proteinase inhibitors isolated from egg-white of birds)<sup>3</sup>, the protein sequence of the reactive centre region has undergone much more rapid evolution than the rest of the molecule. As it is known from *in vitro* biochemical studies that amino-acid substitutions in this region can completely alter the substrate specificity of the inhibitor, the biochemical function of these inhibitors must be evolving rapidly. One of the most remarkable features of these data is the way in which the acceleration of the rate of evolution is confined to the functionally active region. If our current understanding of the function of these molecules is correct, then these results lead almost inescapably to the conclusion that changes



in the amino-acid sequence of the reactive centre regions have been selected for.

During attempts to unravel the complexities of the array of serine proteinase inhibitors found in mammalian blood plasma, it had earlier been found<sup>5</sup> that the gene encoding mouse contrapsin, an inhibitor active against trypsin and similar proteinases, was most closely similar in sequence to a gene in humans that encodes an inhibitor with a quite different specificity:  $\alpha_1$ -anti-chymotrypsin. Similarly, early studies<sup>6</sup> on ovomucoid had shown that the substrate specificity for this inhibitor could be quite different in closely related species. Now, for both gene families, more extensive evolutionary comparisons have been completed.

Contrapsin is now known to belong to a multigene family that encodes at least two classes of messenger (m) RNA. Hill and Hastie in this issue<sup>2</sup> show that a 1.8-kilo-base (kb) mRNA that encodes contrapsin in the mouse is also present in the rat. On the basis of the pattern of expression and on DNA sequence similarity, these mRNAs are probably encoded by the equivalent gene in the two species. A 2.2-kb mRNA from this gene family is also present in both species, and is probably equivalent in each, on similar grounds. Hill and Hastie located the reactive centre region of the contrapsin molecule by aligning the DNA sequence with human  $\alpha_1$ -anti-chymotrypsin. To consider the evolution of these sequences and their human homologue in detail, the nucleotide sequences can be divided into three

regions: the 5' coding region (nucleotides 1-515, codon 175); the reactive centre (nucleotides 516-573, codons 176-191); and the 3' region following the reactive centre. There are stretches of considerable similarity between the sequences of all the genes examined, particularly in the 5' region. Here there are stretches of almost 20 amino acids that have hardly diverged among all four sequences

some debate, it is nevertheless clear that the distribution of amino-acid substitutions along the 51-residue sequence is quite unlike that found in almost all other proteins. In particular, when I examined data from ref. 3, I found the mean number of amino-acid differences in this collection of sequences at those sites which contact the proteinase to be almost double the mean for the whole domain — a statistically

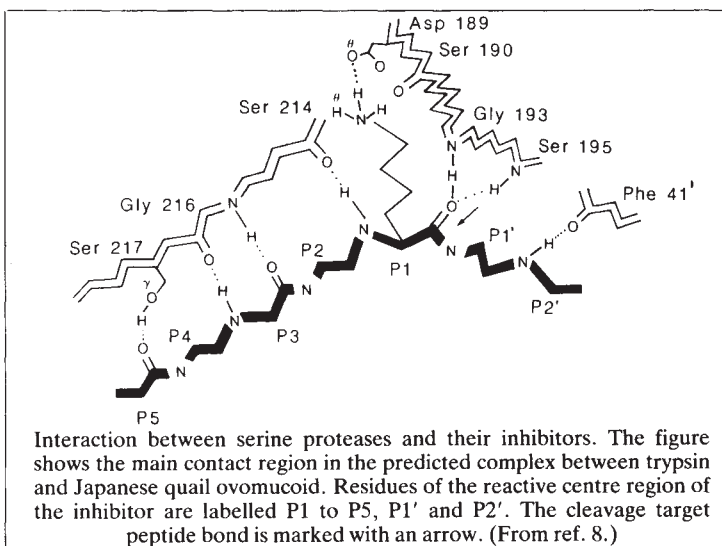
highly significant difference.

There is no doubt about the biochemical importance of the highly variable residues in these serine proteinase inhibitors, especially for the ovomucoids where the enzyme-inhibitor complex has been studied directly by X-ray crystallography. Mechanisms for generating sequence variation other than nucleotide substitution, such as gene conversion, can be excluded in both cases because of the striking difference in the rate of evolution of residues in the reactive centre and their immediate neighbours which do not contact the proteinase.

The simplest explanation for these observations seems therefore to be that new amino-acid sequences in the reactive centre regions have from time to time been favoured by natural selection (and so *s* is positive). But in neither case is the potential selective agent known. The widespread occurrence of serine proteinases makes this a difficult task. Hill and Hastie suggest<sup>2</sup> that the substrates for contrapsin-like molecules are exogenous proteinases synthesized during the course of infection by parasites. This is an interesting proposal as it offers an immediate explanation for the high rate of change. As Haldane originally pointed out<sup>7</sup>, specific host-parasite interactions of this type can result in frequency-dependent selection favouring the maintenance of several alleles of the proteinase of the invading parasite with different specificities in the reactive site. Each new allele of the parasite enzyme would generate a new selective challenge to the host — an evolutionary 'race' begins. Although other explanations have been postulated<sup>3</sup> they do not contain this powerful force for the generation of variability. □

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(mouse contrapsin, rat 1.8-kb sequence, rat 2.2-kb sequence and human  $\alpha_1$ -anti-chymotrypsin). The 3' region also shows considerable similarity among these sequences at both the nucleotide and amino-acid level. But there is a remarkable degree of divergence in the reactive centre region. In only one codon out of sixteen is the same amino acid found in more than two of these sequences. Analysis of the relative rate of synonymous versus non-synonymous substitutions is also revealing. In region 1, the 5' end of the gene, the ratio resembles that of globin genes with synonymous nucleotide substitutions occurring 2-3 times faster than replacement substitutions. Within the reactive centre, however, the frequency of replacement substitutions per replacement site rises to equal the frequency of synonymous substitutions per synonymous site in the 3' region and to exceed that for the 5' coding region. Remarkably, the extent of the region of divergence exactly coincides with the reactive centre, which in fact is flanked by fairly well-conserved sequences.

In the case of ovomucoids, there can be up to three domains containing reactive centres, and in some cases these have evolved different specificities within the same molecule. Laskowski and colleagues have now obtained<sup>3</sup> the amino-acid sequence of the third domain of ovomucoid from 101 species of birds. Although detailed analysis of the rates of evolution of these sequences depends on the phylogenetic framework within which the data are interpreted, and this is currently a topic of



### 100 years ago

SOME years ago I was about to drown a terrier pup of about a month old. I held it across the palm of my open hand over a large tub of water. It lay quite still on my hand as I gently lowered it. When within 4 inches of the surface, but not yet touching the water, it deliberately began, and continued as long as I held it there, the paddling motion with its feet peculiar to dogs when swimming, and quite unlike that of walking, although I am perfectly certain this puppy had never seen or touched water before. We know almost all animals swim when first placed in water, but how could this puppy know before it touched the water that this peculiar action would be necessary? Has a similar case been observed by any of your readers?

Birmingham, February 17 D.W.C.  
From *Nature* **35**, 392; 24 February 1887.

## Protein transport

# From organelle to organelle

Regis B. Kelly

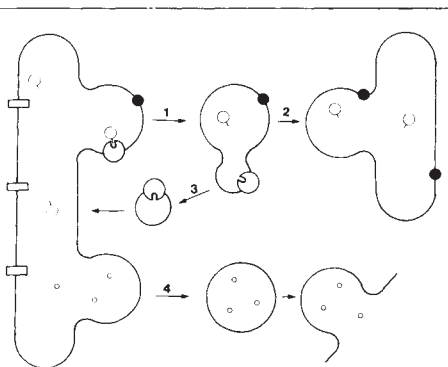
DOMAINS that determine where a protein will be located in the cell, as opposed to what it will do, are called sorting domains (see figure). The domain specifying delivery from the Golgi complex to the vacuole in yeast cells has now been shown<sup>1,2</sup> to be a short contiguous sequence of amino acids at the amino terminus of the protein. Although contiguous amino-acid sequences are known to direct proteins from the cytoplasm to intracellular organelles such as the endoplasmic reticulum or the mitochondria, this is the first example of a sorting domain that specifies movement

the hybrid protein goes to the vacuole<sup>1</sup>. Mutational studies<sup>2</sup> show that the first 10 amino acids at the amino terminus of procarboxypeptidase are sufficient to specify the location of cell vacuoles. When this sequence is mutated, or the sorting machinery is defective, vacuolar enzymes escape from the cell by the pathway normally taken by secreted proteins. Because no sorting information is apparently required for this secretion pathway, computer-friendly biologists now call it the default pathway. Work in the laboratories of Stevens (University of Oregon,

lates inside acidic compartments in the cell, and has a dinitrophenol (DNP) group that can be recognized by DNP-specific antibodies. The molecule also has an amino group that allows gluteraldehyde fixation. By quantifying the binding of anti-DNP using gold-conjugated protein A or secondary antibodies, it is possible to make a semi-quantitative measurement of the pH of intracellular organelles. In insulin-secreting endocrine cells, the condensing vacuoles to which proteins are delivered immediately on leaving the Golgi cisternae are much more acidic than the cisternae themselves<sup>3</sup>. Because low pH facilitates the proteolytic conversion of proinsulin to insulin, acidification in this case could be necessary solely to ensure that proteolysis is a late, post-Golgi event. Exocrine cells, however, do not require proteolysis of their secretory granule enzymes and, unlike endocrine cells, they do not have secretory granules with a low internal pH. When Orci and colleagues examined exocrine cells with DAMP, as they report in this issue<sup>4</sup>, the secretory granules are not acidic as expected, but the same acidification increase is seen from Golgi cisternae to immature secretory granule.

Several cellular processes could be responsive to a pH gradient from the Golgi complex to the immature vesicles of regulated secretory cells. The hypothesis with perhaps the strongest appeal to cell biologists is that the pH gradient allows vectorial, one-way traffic of secreted proteins from the Golgi complex to immature secretory granules. In this hypothesis, vectorial sorting into the regulated, secretory pathway would parallel vectorial delivery of ligands by receptor-mediated endocytosis<sup>7</sup>, and delivery of newly synthesized lysosomal enzymes from Golgi lysosome to lysosomal precursor<sup>6</sup>. To achieve vectorial transport during endocytosis and lysosome biogenesis, receptors carry ligands from a donor organelle to an acceptor organelle where the acidic interior promotes ligand dissociation (see figure). The empty receptors then recycle to the donor membrane while the ligand proceeds to the lysosome. Energy that is needed to ensure vectorial one-way traffic is provided by the ATP-driven proton translocases that generate the pH gradient. Because the mannose phosphate receptor and receptors involved in receptor-mediated endocytosis show pH-dependent ligand association, acidotropic drugs which destroy pH gradients block vectorial delivery. An old observation<sup>8</sup> suggested that delivery of hormone to secretory granules in endocrine cells was similarly blocked by acidotropic molecules. This result and that showing the accumulation of DAMP in immature secretory granules are consistent with a model in which a pH-sensitive, recycling receptor executes one-way vectorial traffic of

Steps in organelle-to-organelle sorting. Any newly synthesized protein destined either for secretion or for one of the membrane-bounded compartments of the cell is directed first to the Golgi complex. In the Golgi complex, the proteins are sorted, by unknown mechanisms, to their correct intracellular destinations. If a newly synthesized protein lacks sorting information it leaves the Golgi complex and goes directly to the surface of the cell by a default pathway. Proteins destined for delivery to the lysosomes and, in certain secretory cells, for delivery to the secretory granule may be sorted out of the default pathway by the above sequence of steps. (A parallel sequence of steps is hypothesized for delivery of proteins from the cell surface to the endosomes.) 1, Membrane proteins, including those that are receptors for ligands with sorting domains, are segregated away from resident membrane proteins of the donor organelle. 2, In a compartment with an acidic interior, ligands dissociate from receptors. Free ligands are targeted to the donor organelle. 3, Empty receptors are segregated from the other membrane proteins and soluble ligands and return to the donor organelle. 4, Soluble proteins that lack sorting domains can escape by a non-selective default pathway. If steps 2 and 3 are blocked with an acidotropic drug, ligands with sorting domains exit through the default pathway.



between organelles. R. Anderson and colleagues, in a separate set of observations reported in ref. 3 and on page 77 of this issue<sup>4</sup>, find that the lumen of Golgi-associated vesicles is most acidic in the region where newly synthesized secretory proteins leave the Golgi area for mature secretory vesicles. The observations give further support to the notion that unidirectional flow of proteins from one organelle to another involves pH-sensitive receptors. Yeast-cell mutants deficient in interorganelle sorting<sup>1,5</sup> may allow direct testing of such sorting hypotheses.

Mammalian cells most often use a mannose phosphate receptor to target newly synthesized lysosomal enzymes to the lysosome<sup>6</sup>. Yeast cells use a simpler sorting mechanism, that does not require carbohydrate, to target digestive enzymes to the vacuole, an organelle analogous to the lysosome of animal cells. When as few as 50 amino acids from the amino terminus of procarboxypeptidase Y, a vacuolar enzyme precursor, are fused to the amino terminus of invertase, a secreted protein,

Eugene<sup>5</sup> and of Emr (Caltech)<sup>1</sup> has made elegant use of the diversion of mis-sorted vacuolar enzymes to the default pathway to identify at least 14 complementation groups involved in targeting vacuolar enzymes. Such mutations need not affect vacuolar membrane protein localization (J. Rothman and T. Stevens, personal communication), implying that sorting of membrane and soluble vacuolar proteins involves at least partially different mechanisms.

What molecular machinery is required to transfer vacuolar enzymes from Golgi complex to vacuole? A good bet is that the sorting machinery will use acidity differences between intracellular organelles. Acidity differences have now been discovered<sup>3,4</sup> between regions of the Golgi complex in at least two cell types that sort secreted proteins into the regulated secretory pathway. The key advance in these studies is the use of a novel membrane-permeable acidotropic molecule, 3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine (DAMP). This molecule accumu-



endocrine hormones and zymogens into their respective secretory organelles. It would be very exciting if vacuolar sorting in yeast cells, where mutants are now available, uses the same basic pH-sensitive recycling mechanism, and perhaps also clathrin-coated segregation of receptors, to sort newly synthesized proteins out of the default pathway. If so, cell biologists will have yet another unifying hypothesis to simplify their lives. □

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## Mid-ocean ridges

# A magma chamber observed?

Charles H. Langmuir

A SUBSTANTIAL step towards clarification of the controversy about magma chambers has been taken in the past year by a team of investigators from three US oceanographic institutions who carried out a comprehensive multi-channel seismic survey of the East Pacific Rise. On page 35 of this issue<sup>1</sup>, Detrick *et al.* report the preliminary results of the survey. These results constrain for the first time the distribution of magma chambers along the East Pacific Rise, and provide the best constraints yet on the width of the axial magma chamber.

The concept of a magma chamber, where magma is stored before its eruption from volcanoes, has been central to petrological thought for much of this century. The concept is particularly attractive for the 50,000-km-long string of volcanoes that make up the ocean ridges, where the plates of the Earth spread apart at rates between 1 and 20 cm per year. Magma rises from the mantle to fill the crack created by spreading, and this continual supply of magma has been suggested to supply a large 'steady-state' magma chamber that would exist perpetually along the global system of ocean ridges. This model has been called the 'infinite onion' model of ocean ridges<sup>2</sup>, because the magma chamber peels off solid layers at its edges while continually growing from its centre. Scientists studying ophiolites, old ocean crust exposed on land, have suggested that ocean ridge magma chambers can be as much as 30 km wide, in apparent agreement with the infinite onion model<sup>3</sup>.

But there has been a contradiction between this conceptual model of ocean ridges with large, steady-state magma chambers and the actual evidence from geophysical and geochemical studies. Indeed, some have suggested, on the basis of both petrology<sup>4,6</sup> and geophysics<sup>6,7</sup>, that magma chambers are small and ephemeral rather than large and steady-state.

The multi-channel technique reveals reflecting horizons within the ocean crust. A magma chamber would be a zone of low-velocity liquid surrounded by higher-

velocity rock, and the top of the chamber should cause a bright reflection in the seismic records. The reflection should also show a phase reversal, as the liquid magma chamber would have lower seismic velocities than the solid lid, in contrast to the usual increase in velocity with depth observed in solid ocean crust. A reflection with the right characteristics had previously been observed<sup>8</sup> on profiles across the axis of the East Pacific Rise, but the reflector was weak and there was no information about its continuity along the axis.

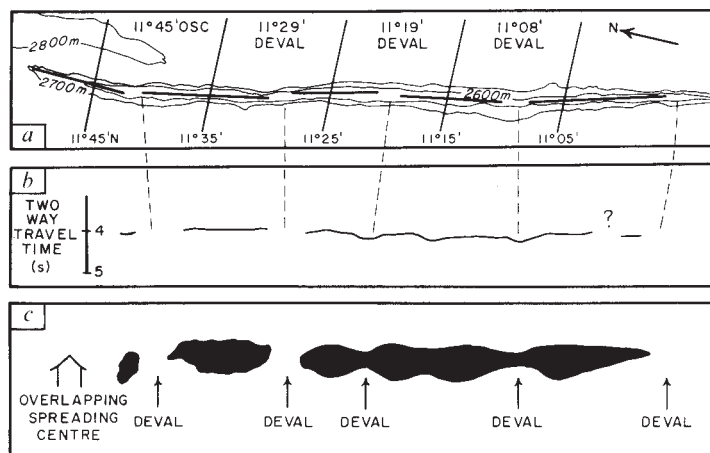
The question of continuity is of particular interest because of recent results suggesting that the East Pacific Rise is petrologically segmented at small 'deviations from axial linearity' (devals) as well as at transform faults and overlapping spreading centres<sup>5</sup>. Through the use of the SeaBeam echo-sounding system, Detrick and co-workers were able to drive the ship along instead of across the axis of the East Pacific Rise, and for the first time to determine the continuity of the axial reflector. They found it to be present about 60 per

cent of the time, and the width of the reflector, where present, usually to be less than 3-4 km. The reflector is discontinuous across overlapping spreading centres and transform faults, and in some (but not all) cases is discontinuous or shows marked dips at devals.

It is tempting to associate the axial reflector with the magma chamber in the infinite onion model. If the reflector is a magma chamber, then the seismic data imply that the magma chamber is an order of magnitude smaller than the ophiolite-based model. Also, because the magma chamber is present beneath only a little more than half of the length of the East Pacific Rise studied, it cannot be a steady-state feature except possibly in limited areas.

Several ambiguities remain in the interpretation of what the reflector actually represents. The first ambiguity derives from the fact that the reflection is from an interface, and the data processing so far provides little information about the nature of the material beneath the interface, expect that its velocity is often lower. The seismic results do not really distinguish between a mostly liquid magma and a mostly solid mush. Second, the reflector is not always phase-reversed, and is sometimes observed far off-axis where Detrick *et al.* suggest it results from the 'frozen top' of the magma chamber. Presumably, some of the reflector along the axis that does not show a phase reversal could be a frozen top as well. On the other hand, the reflector is so narrow that some of the gaps in it might be a consequence of the ship straying slightly off-axis or of local roughness in topography.

Two ways to learn more about the material properties of the crust where the axial reflector is observed are by expanding spread profiles and by studies using ocean-bottom seismometers. Detrick *et*



Possible distribution of magma chambers along the East Pacific Rise. *a*, Bathymetry of the East Pacific Rise in plan view between 10°50'N and 1°45'N<sup>1</sup>. The Clipperton transform fault is another 60 km to the south, at 10°15'N. Bold straight lines indicate individual volcanic segments separated by devals. *b*, Depth (in seconds of two-way travel time) and location of the axial reflector identified by the seismic survey<sup>1</sup>. Devals in the bathymetry and corresponding gaps or dips in the reflector are connected by the vertical dashed lines. *c*, Vertical section of the possible distribution of magma chambers beneath this portion of the East Pacific Rise. The chamber is discontinuous across spreading centres, and may thin or disappear across devals.

*al.*<sup>1</sup> carried out one expanding spread profile on the axis within the region where they report along-axis data, and there is evidence for an extensive zone of low velocity at that one point along the axis. But both axial mush and an axial magma chamber could produce such a low-velocity zone.

Studies using ocean-bottom seismometers potentially hold great promise as a complement to the multi-channel seismic studies, because they can evaluate waves which pass through the low-velocity zone rather than waves that are reflected off it. Bratt and Solomon<sup>7</sup> reported the analysis of seismograms from a three-component ocean-bottom seismometer at 11°20'N, where Detrick *et al.*<sup>1</sup> found there to be a weak axial reflector in the multi-channel seismic data, and where there is a deval<sup>5</sup>. Bratt and Solomon concluded that any magma within the crust in this region must be confined to narrow dykes or small isolated bodies with less than 1 km vertical thickness. Thus, even in regions where the seismic reflector is continuous there may be variations in the crystallinity and thick-

ness of the axial low-velocity zone. An along-axis profile that might satisfy the seismic constraints and be consistent with the inferences from the petrological data is sketched in the figure. Given the new along-strike perspective of the multi-channel data, new ocean-bottom seismometer studies located where the axial reflector is strongest and where it is absent should help to clarify the relationships among the multi-channel seismic reflector, possible axial magma chambers and the chemical systematics of basalts erupted at the East Pacific Rise. □

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## Cell motility

# Microtubule activation of kinesin ATPase activity

Stanley Cohn

UNDERSTANDING the molecular mechanisms responsible for force generation along cytoskeletal elements such as microtubules and actin filaments is central to the study of intracellular movement of organelles. Several proteins have been isolated from eukaryotic cells that interact with these cytoskeletal elements and are found to use ATP to generate motile force. The best-characterized of these proteins, myosin, hydrolyses ATP at a rate directly coupled to motile activity. Thus, the ATPase of purified myosin is low, but is several hundred times higher in the presence of actin filaments<sup>1</sup>. Although purified dynein, another mechanochemical protein, has a substantial rate of ATP hydrolysis, it has recently been shown<sup>2</sup> that this activity can be stimulated by microtubules, the cytoskeletal cofactor of dynein. And new evidence<sup>3</sup> obtained by Kuznetsov and Gelfand shows that kinesin, another force-transducing protein, hydrolyses ATP at rates appropriate for organelle transport along microtubules.

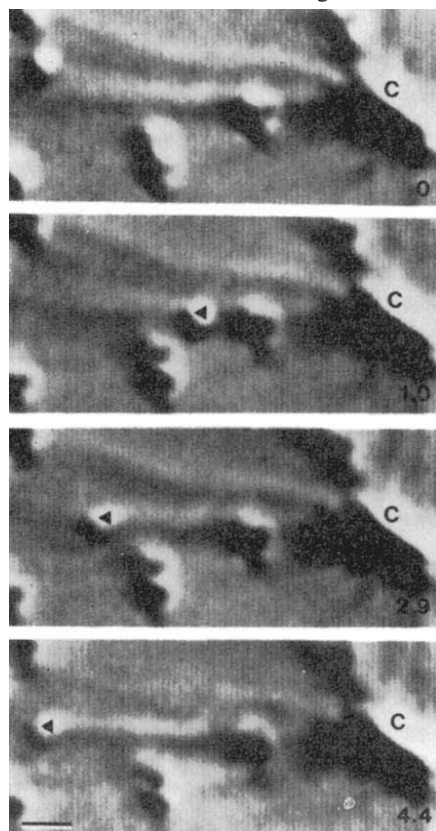
Kinesin has recently been isolated from several cell types where it may participate in organelle transport and mitosis (refs 4,5; see ref. 6 for review). Kinesin has been shown by video-enhanced microscope assay to be capable of producing translocation of carboxylated latex beads along microtubules or of microtubules

over a glass coverslip (see figure). The motility induced by neuronal and sea urchin egg kinesin is totally dependent on the presence of hydrolysable nucleotides, with a maximal translocation velocity of approximately  $0.5 \mu\text{m s}^{-1}$  when in saturating ( $> 100 \mu\text{M}$ ) concentrations of ATP<sup>7,8</sup>. Assuming that each kinesin crossbridge translocates a microtubule or bead a distance of one to two microtubule subunits (about 10 nm), then an active kinesin molecule would be expected to hydrolyse ATP at a rate of around 50 per second, a value that would be similar to the activity of the actin-activated MgATPase of myosin 'crossbridges' (subfragment-1) and that of the microtubule-activated ATPase of dynein. However, both neuronal and sea urchin kinesin, when prepared by a procedure based on the tight binding of kinesin to microtubules in the presence of the unhydrolysable ATP analogue AMPPNP and its subsequent release by ATP<sup>4-6</sup>, do not possess these predicted high rates of ATPase activity.

In their new work, Kuznetsov and Gelfand<sup>3</sup> obtained preparations of bovine brain kinesin that hydrolyse ATP at the high rates predicted from the motility assays. Interestingly, the authors imply that kinesin prepared by the method of binding kinesin to microtubules in the presence of AMPPNP yield fractions with

low ATPase activity. But if they replace AMPPNP with inorganic triphosphate (PPP<sub>i</sub>) in the microtubule-affinity step, they obtain material on ATP release which elutes from a gel-filtration column as a protein complex containing a major polypeptide of relative molecular mass (*M*<sub>r</sub>) 135,000 and minor ones of *M*<sub>r</sub> 45,000-70,000. This protein complex hydrolyses MgATP at a rate of 60-80 nmol min<sup>-1</sup> mg<sup>-1</sup> and is stimulated about 60-fold to 4.6  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  in the presence of microtubules.

Although Kuznetsov and Gelfand did not directly study the motility-inducing activity of their preparations, there are several reasons for believing that their



A series of photographs taken from a time-lapse video sequence showing the distally oriented movement of a kinesin-coated bead (marked with a triangle) along a microtubule which had been nucleated and assembled off an isolated centrosome (C); lower right-hand numbers are seconds after bead attachment. Scale bar, 1  $\mu\text{m}$ . For details see refs 7 and 12.

microtubule-activated ATPase represents the activity caused by kinesin-induced motility. For example, the ATPase activity varies with ATP concentration according to Michaelis-Menten kinetics, with a *K<sub>m</sub>* (the concentration of substrate required for half-maximal activity) for ATP of 12-14  $\mu\text{M}$ . The rate of kinesin-induced microtubule gliding over glass coverslips exhibits a similar variation in this range, resulting in half-maximal velocity at 10-20  $\mu\text{M}$ <sup>7,8</sup>. Furthermore, PPP<sub>i</sub> and AMPPNP, both of which promote stable microtubule-kinesin complexes and in-



hibit kinesin-induced microtubule gliding, were found by Kuznetsov and Gelfand to be competitive inhibitors of the microtubule-activated ATPase. Vanadate and *N*-ethylmaleimide, which are weak inhibitors of kinesin-induced motility<sup>4,8</sup>, are also found to be weak inhibitors of the ATPase activity.

Nonetheless, the implication that the use of AMPPNP in kinesin purification yields protein with inhibited enzymatic activity raises some puzzling questions. For example, the microtubule motility promoted by both squid neuronal and sea urchin egg kinesin purified by the AMPPNP-induced microtubule-binding procedure seems to be very similar to the motility observed using crude preparations that have not been exposed to AMPPNP<sup>4,8,9</sup>. If the ATPase is indeed caused by the kinesin microtubule cross-bridge cycle coupled to motility, then it is difficult to understand how the two kinesin preparations (using PPP<sub>i</sub> and AMPPNP for microtubule binding), having equal ability to induce microtubule translocation, could differ so greatly in their ATPase activities. Also, AMPPNP, like PPP<sub>i</sub>, appears to act as a competitive inhibitor of the microtubule-activated ATPase of kinesin, implying that its inhibitory effects should be reversible with increasing ATP concentration. Moreover, Brady and co-workers<sup>10,11</sup> have prepared microtubules from chick and bovine brains in AMPPNP which contain polypeptides of *M*<sub>r</sub> 130,000 and 124,000, respectively, and hydrolyse ATP at a much greater rate than microtubules prepared in ATP in which these polypeptides were absent. It is therefore seems likely that other factors, such as removal of ATPase

inhibitors or retention of other regulatory cofactors during purification or the exact conditions of their ATPase assay, also contribute to Kuznetsov and Gelfand's success in isolating enzymatically active kinesin.

The work of Kuznetsov and Gelfand is a significant step in the study of kinesin-induced motility. Measurements of ATPase activity, together with the video-microscope motility assays, should allow the mechanochemical cycle of kinesin to be studied in greater detail, as well as being extremely useful in the development of probes (for example, antibodies) that inhibit kinesin enzymatic activity and that can be used for investigating biological functions of kinesin *in vivo*. The purification protocol developed by Kuznetsov and Gelfand also represents one other advance: the cost of one gram of PPP<sub>i</sub> is about \$13 whereas an equivalent amount of AMPPNP costs \$1,407. □

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## History of science

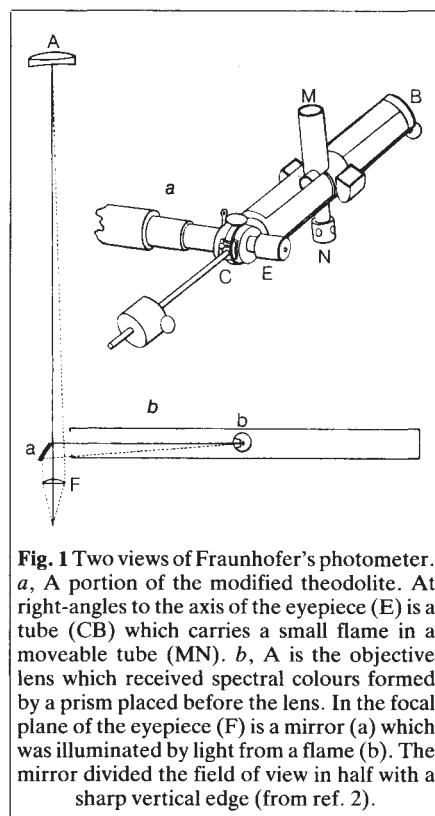
# Joseph Fraunhofer (1787–1826) and heterochromatic photometry

Paul L. Pease

JOSEPH Fraunhofer, born 200 years ago on 6 March 1787, was probably the first to use the criterion of a minimally distinct border to equate the brightness of two heterochromatic fields. The purpose of heterochromatic photometry is to determine at what radiance lights of different chromaticity have an equal visual effect. Results obtained with the minimally distinct border and other procedures of heterochromatic photometry are central to our understanding of the inter-relationships of physical, physiological and perceptual variables in the processing of visual information. Although Fraunhofer may not have been the very first to use the minimally distinct border, his contribution in the early part of the nineteenth century

has been largely unrecognized.

Fraunhofer published two papers<sup>1,2</sup> in 1823 and 1824. The second of these reports his experiments on the brightness of different colours in the spectrum and his measurements of chromatic aberration of the eye. Although Fraunhofer is best known for his contribution to optics, I discuss here his measurements of the brightness of the spectrum and the method he used to obtain them. Why did Fraunhofer want to know, as he stated on page 32 of ref. 2, "the intensity of each colour in the spectrum, or in what ratio the impression of any colour of the spectrum is stronger or weaker than that of another colour?" He had worked out the design for achromatic doublets for telescopes



**Fig. 1** Two views of Fraunhofer's photometer. *a*, A portion of the modified theodolite. At right-angles to the axis of the eyepiece (E) is a tube (CB) which carries a small flame in a moveable tube (MN). *b*, A is the objective lens which received spectral colours formed by a prism placed before the lens. In the focal plane of the eyepiece (F) is a mirror (a) which was illuminated by light from a flame (b). The mirror divided the field of view in half with a sharp vertical edge (from ref. 2).

that were, of course, intended to be used by the eye. He was interested in certain ocular parameters, such as axial chromatic aberration and spectral sensitivity, and he reasoned that the residual chromatic aberration of an achromatic lens would be bothersome to an observer if any residual aberration occurred in those parts of the visible spectrum that appear the brightest. He wrote on pages 31 and 32 "... the aberration of the yellow rays, for example, which have the greatest brightness, will produce in the ratio of their intensity a worse effect than the violet ones, if the aberration for the latter is of the same magnitude".

To measure the brightness of the different parts of the spectrum, Fraunhofer used a theodolite, a telescope used for the measurement of angles (Fig. 1). The light of different colours of the spectrum of the Sun were obtained by a glass prism and viewed through the telescope. In the focal plane of the ocular, he "applied a small plain metallic mirror, the edge of which being well defined" (page 32). The mirror was placed at 45° to the axis of the telescope that bisected the field of view. A tube at right angles to the ocular contained his standard source — a small aperture placed before an oil flame. He varied the illuminance of the flame at the mirror by changing the distance of the source from the mirror. Fraunhofer had, in fact, constructed a visual photometer with a circular bipartite field, one half illuminated by his standard lamp and the other by the dispersed spectrum of sunlight. Knowing the distance of the lamp from the mirror

and applying the inverse square law, he obtained measurements of the relative brightness of different spectral lines. The criterion he used to compare the two halves of the field which, of course, had different chromaticities, was a minimally distinct border. "Though at first it appears difficult to compare the light of two different colours, yet it becomes easy by a little practice. The intensity of the light of the mirror approaches more to that of any colour in the spectrum, if at the same position of the eye-glass its vertical margin is less distinct" (page 32).

Fraunhofer reported the results of four experiments. "Though the experiments were made in clear weather, and at noon, I sometimes perceived, in the course of the observations, a slight change in the density of the light which the prism received. The differences in the four sets of experiments may have been partly owing to this change, and the flame may also have changed its intensity in the course of the observations" (page 33). This, then, is his explanation of why he obtained variable results from one experiment to the other. I emphasize this point here because of Helmholtz's later criticism (see below). Fraunhofer was aware that his results were relative measurements because he noted that the brightness of different parts of the spectrum was dependent on the source of light. He was also well aware that his results could have been influenced by variability in the absolute intensity of either the Sun or the flame.

I next re-examined the 1968 paper<sup>3</sup> of Boynton and Kaiser which I believe to be the first modern account of the minimally distinct border criterion. Were these authors aware of Fraunhofer's contribution? They state "Our search of the literature has found no evidence of such an experiment; nearly all writers refer to an equal-brightness criterion, making no reference to the quality of the border between the

fields" (page 368). The authors cite ref. 4 and state: "Helmholtz mentions that Fraunhofer wrote of a minimally distinct border that could be achieved between areas of different color, but Fraunhofer was not concerned with photometric measurement". I believe that two errors should be corrected: first, the statement "Fraunhofer was not concerned with photometric measurement" contradicts Fraunhofer's own reason for measuring the brightness of different colours; and second, Helmholtz and/or Southall<sup>4</sup> erred in saying "The numerical results of Fraunhofer's measurements of the brightness of the different portions of the spectrum agreed very poorly, mainly, no doubt because he was ignorant of the effect of the absolute intensity on the relative luminosity of the colours". On the contrary, Fraunhofer wrote about how variation in the absolute intensity could have affected his results.

It is of interest to know how the results of Fraunhofer's experiments compare with more recent measurements. Leitner has described<sup>5</sup> Fraunhofer's contribution but, like Fraunhofer, did not correct the data for the irradiance of the Sun. I there-

fore corrected Fraunhofer's data for the absolute irradiance of the Sun according to ref. 6. The corrected measures are shown in Fig. 2 together with more recent heterochromatic data<sup>7</sup>. Apart from the extremes of the spectrum, it is clear that Fraunhofer had it about right.

Certainly the knowledge that Fraunhofer used the minimally distinct border criterion more than 150 years ago does not diminish the significance of the contributions of Boynton and Kaiser and others (see refs 7-9). But the anniversary of Fraunhofer's birth is an appropriate time to set the record straight. □

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## Protein – DNA interaction

# Another folding problem?

Timothy J. Richmond

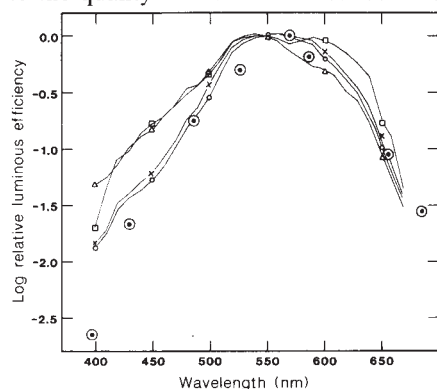
Now that the first structure of a protein–DNA complex at atomic resolution has been solved by X-ray crystallography and with several other structures on the way, the variety of means by which protein molecules recognize the DNA double helix is becoming clearer. McClarin *et al.* have recently reported<sup>1</sup> the X-ray structure of a bacterial restriction endonuclease, *EcoRI*, bound to a short DNA fragment containing the recognition site of the enzyme. The enzyme does not cleave the DNA because  $Mg^{2+}$ , which is essential for catalysis, is left out of the crystallization mixture. Comparison of this structure with that for the free DNA, which is known<sup>2</sup>, reveals that the protein induces distortions or kinks in the DNA that are required for formation of the specific complex.

The *EcoRI* protein binds DNA as a dimer, giving the complex a dyad axis of symmetry which coincides with a crystallographic dyad (see figure). The DNA double helix is unwound at this central dyad position by about 25° because of the interactions which are made between its phosphate groups and major groove and the chains extending from two  $\alpha$ -helices on each protein monomer. The side chains of one glutamate and two arginines from each subunit make six hydrogen bonds with five base pairs in the hexanucleotide recognition site. Each of these

side chains and base pairs participates in two hydrogen bonds. The unwinding of the DNA that permits the formation of all these hydrogen bonds may itself be sequence-dependent. A synthetic homologue of the DNA fragment TCGCGAATTCGCG used by McClarin *et al.* in which the central adenines are replaced with 2,6-aminopurine might provide a test of his hypothesis: it would introduce a base pair that mimics the Watson–Crick hydrogen bonds of G–C while preserving the positions of hydrogen-bond donors and acceptors in the major groove.

The irregular conformation of the DNA centred on the molecular dyad is not a kink in the sense proposed originally<sup>3</sup> by Crick and Klug. These authors suggested that DNA might not bend smoothly to supercoil at the radius of about 45 Å found in the nucleosome, but instead kink at regular intervals between straight segments of B-form double helix. In their model for a kink, contacts between the flat surfaces of neighbouring base pairs were broken and replaced by interactions with protein or water. The DNA seen in the X-ray structure of the nucleosome core particle is actually bent tightly in several places, but the base stacking is not yet clear<sup>4</sup>.

McClarin *et al.* broaden the definition of a kink to include twist as well as curvature distortions of idealized B-form DNA.



**Fig. 2** Mean results of Fraunhofer's four experiments (corrected for the spectral irradiance of the Sun) compared with other measures of relative luminous efficiency (from ref. 7). Symbols: ⊙, Fraunhofer's data; ○, heterochromatic flicker photometry; ×, minimally distinct border; △, step-by-step; □, direct comparison. The last two are measures of direct heterochromatic brightness matching.



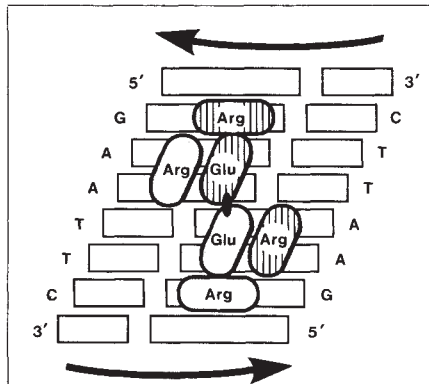
Because the DNA fragment on its own does not untwist<sup>2</sup>, they call the distortions induced in it by *EcoRI* binding type I and type II 'neokinks'. In contrast to the untwisting that characterizes the type I neokink, the type II neokink, which occurs at the base step flanking the hexanucleotide recognition sequence, is characterized predominately by a bend of  $30 \pm 10^\circ$  into the DNA minor groove. When this structure has been fully refined, it might be possible to measure the base-pair unstacking by comparing the difference in the surface accessibility of the DNA in the complex with that for the DNA alone.

The two  $\alpha$ -helices in each subunit that contact the DNA are part of a domain in which several  $\alpha$ -helices surround a five-stranded  $\beta$ -sheet. The major  $\alpha$ -helical regions are oriented with their electro-positive amino ends pointing at the negatively charged DNA molecule, compensating for the negatively charged glutamate side chains used in the hydrogen-bonding scheme. A smaller domain, consisting mainly of a two-stranded  $\beta$ -sheet, wraps around the DNA along the major groove. It is required for catalytic activity, but is not essential for specific recognition as it can be removed proteolytically without affecting selective binding. This domain apparently contributes to the formation of the type II neokink, but it is unclear whether this flexing of the DNA phosphate chains is required for catalysis. These details may soon emerge, as  $Mg^{2+}$  can be diffused into the crystals to activate the cleavage reaction without disrupting the crystals.

Does the *EcoRI* study have implications for other protein-DNA complexes? The *EcoRI* protein displays a high degree of specificity for its six base-pair recognition site, to which it binds at least  $10^4$  times more tightly than to random sequences. The *cro* repressor<sup>5</sup>,  $\lambda$ -repressor<sup>6</sup>, catabolic activator protein<sup>7</sup>, *trp* repressor<sup>8</sup> and similar proteins are in another class of highly specific prokaryotic DNA-binding proteins for which X-ray structures are available at high resolution, but lack their DNA counterparts. Model building from the protein structure alone and biochemical experiments could only hint at the DNA contacts responsible for specificity. The low-resolution structure of the 434 repressor-DNA complex suggests that rigid, unbent, B-form DNA, as used in the model-building studies, may not adequately represent high-resolution details<sup>9</sup>. Fortunately, several X-ray investigations of cocrystals containing these proteins bound to DNA and diffracting to atomic resolution are in progress.

All proteins that bind functionally to the DNA double helix show preferences for specific sequences, even though they may not be highly specific like the *EcoRI* protein. Pancreatic DNase I, for example, for which specificity is not a requirement,

cuts the phosphate ester bonds of the *EcoRI* oligonucleotide at rates that differ by more than 100-fold<sup>10</sup>. Cleavage of larger DNA substrates<sup>11</sup>, and the atomic resolution structure of the DNase I enzyme itself<sup>12</sup>, suggest that the discrimination arises largely in the binding step and is caused by the snug fit of a tyrosine side chain into the minor groove of the DNA. The width of the groove is thought to depend on base sequence, but perhaps the enzyme causes additional variations when



The hexanucleotide-recognition DNA unwinds to accommodate the hydrogen bonds between it and the *EcoRI* protein. Part of the DNA fragment crystallized with the nuclease is shown schematically, looking into the major groove down the molecular dyad axis. The arrangement of the hydrogen-bonding side chains, three from each subunit of the *EcoRI* dimer, is indicated. The DNA in the complex is twisted and bent substantially<sup>1</sup> compared with the same sequence in the free state<sup>2</sup>.

it binds. Even the nucleosome, which packages more than 90 per cent of the genome of eukaryotic cells, favours certain DNA sequences. On average, a nucleosome will occur roughly every 200 base pairs, but within this length it may be positioned quite precisely, preferring certain sequences and excluding others at distinct locations along the helical ramp formed by the histone core<sup>13</sup>. As much as a 100- to 1,000-fold preference may occur within one nucleosomal repeat. The nucleosome present at the beginning of the sea urchin 5S RNA gene, for example, is particularly well positioned *in vivo* as well as *in vitro*<sup>14,15</sup>. Approximately 160 base pairs are folded around the histone octamer core of the nucleosome; the bending and twisting required to fit the DNA to the protein surface may be energetically more favourable for certain base sequences than for others, such that their combined locations determine nucleosome position and stability. Alternatively, the sequence preference may be controlled locally by the H3 histone dimer in the region where the dyad axis passes through the centre of the DNA<sup>4</sup>. The details of H3-DNA contact are obviously different from those for *EcoRI*, but the twist imparted to the DNA may be similar. Overall, specificity at any level may require sequence-dependent

alterations in the DNA structure induced by protein binding.

Is the specificity of interactions between protein and DNA governed by simple rules, such as a list of hydrogen-bond match-ups between the two components as was predicted for the 'two-helix motif' proteins, or is the problem comparable in complexity with the protein-folding problem, where a description of the combined intermolecular forces so far eludes us? The answer is apparently yes to both possibilities. The beauty of the X-ray crystallographic solution of the *EcoRI*-DNA complex<sup>1</sup> is that it not only shows the exact mapping of the hydrogen bonds between protein and DNA, but it also reveals substantial distortions of the canonical B-form DNA structure required for the formation of these bonds.

Unravelling the relevant structures by X-ray analysis has dramatically increased our understanding of protein-DNA interactions; there is much more to look forward to. We now know the structures of several types of protein templates to which DNA must conform to be recognized: the restriction enzymes with homology to *EcoRI*; the two-helix motif proteins; pancreatic DNase I; the histone octamer; *Escherichia coli* DNA polymerase 1<sup>15</sup>; and the bacterial chromosomal protein HU<sup>16</sup>. Eventually, knowledge of the structure of DNA-binding protein may allow the prediction of which base sequence will be recognized or, at least within a given class of template, it may be possible to design an altered specificity. On the other hand, the structures of several of the two-helix motif proteins have not yet led to a unique model for the DNA complexes of these systems. Even with the lessons learned from the *EcoRI*-DNA structure, the adventurous model builder lives dangerously — should the DNA be twisted or bent? Perhaps an allosterically regulated DNA-binding protein, such as the *trp* repressor, will provide a few more clues<sup>6</sup>. Comparison of the structures for the non-specific and specific DNA complexes may reveal the limits on how far DNA can twist and bend to conform to a protein. □

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## Ozone climatology

## More news from Antarctica

Susan Solomon

MUCH effort has been directed towards obtaining a better understanding of the spectacular modification of the Antarctic ozone layer called the ozone 'hole', a precipitous drop in total ozone abundance that has appeared over Antarctica each austral spring (September and October) since about 1975. Forty-five papers on the subject appeared in a special issue of *Geophysical Research Letters* in November 1986. The National Ozone Expedition observational campaign was carried out by 18 scientists (of which I was one) at the National Science Foundation's McMurdo Station during austral spring 1986, and its preliminary results were discussed<sup>1</sup> in a recent News and Views article. The first paper on the results from the expedition, by Hofmann and collaborators, appears on page 59 of this issue<sup>2</sup>.

The phenomenon of the ozone hole was first identified<sup>3</sup> by scientists from the British Antarctic Survey, based on a 30-year record of total ozone measurements at Halley Bay, Antarctica. These data suggested<sup>4</sup> that the total ozone concentration should remain rather constant during Antarctic spring, and indeed, such a seasonal cycle was observed<sup>5</sup> before about 1975. The recent data display a different, clearly anomalous, seasonal trend. Further, the magnitude for the spring decline is generally greater from one year to the next, although some year-to-year fluctuations are also observed. Contemporary total ozone abundances in Antarctica in October are only about half as large as those obtained<sup>3</sup> between 1950 and the early 1970s. This is the largest, statistically significant perturbation to a monthly averaged ozone climatology ever recorded.

It is beyond the scope of this article to review the current status of the rapidly growing subject of research on the ozone hole. Instead I will discuss representative rather than comprehensive references. There are several plausible hypotheses<sup>5-8</sup> that seek to explain the observed behaviour of the ozone column. Each results in total ozone changes that are broadly consistent with observations, but each has rather different implications for dynamical tracers; for other chemical species such as nitrogen dioxide, chlorine nitrate or chlorine oxide; and for the vertical structure of the ozone column change. Detailed observations of these parameters and further laboratory studies will be needed before a definitive theory of the ozone change can be advanced. Recent studies provide some interesting insights and apparent contradictions in the behaviour of the Antarctic stratosphere.

It is of great interest to ascertain whether a temperature trend has accompanied the observed ozone trend, because both dynamical and chemical processes (particularly those occurring in polar stratospheric clouds<sup>5,6</sup>) are expected to be closely linked to temperatures. Meteorological analyses<sup>9</sup> based mainly on satellite data display a temperature change as large as 18 °C based on a rather limited time series dating only from 1979 to 1985. On the other hand, a detailed analysis<sup>10</sup> of radiosonde data encompassing the period 1958–1985 suggests a much smaller, but statistically significant trend of about 6–8 °C. The differences between the two datasets are thus quite large and perhaps contradictory, requiring further investigation. Such a trend would be of great importance if substantiated, as it would represent a significant departure from temperature climatology, just as the ozone hole itself is a verified and extreme departure from ozone climatology. Could such a temperature change be unambiguously assigned as the cause, or as an effect, of the observed change in ozone? Radiative transfer theory suggests<sup>11</sup> that the observed change in ozone could lead to a 5–15 °C change, but it seems clear that a significant change in dynamics could yield perturbed temperature and possibly ozone distributions.

A useful database on Antarctic nitrogen dioxide from studies<sup>12</sup> by a New Zealand group, is also available. These data display unusually low abundances during Antarctic spring. Like ozone, the seasonal cycle of nitrogen dioxide is in marked disagreement with conventional theory<sup>5</sup>, demonstrating that the chemistry of the Antarctic spring stratosphere is highly anomalous. Further observations of chemical species are needed to determine whether these anomalies are directly associated with the ozone anomaly.

The paper by Hofmann and colleagues in this issue<sup>2</sup> reports a series of ozone-sonde observations carried out as part of the National Ozone Expedition. These data provide several important insights into the problem of the ozone hole. Perhaps the most significant is their demonstration that the seasonal decline in the total ozone column is largely confined to the region from about 12 to 20 km. The observed timescale for the ozone change was very short, only about a month. The bulk of the depletion of the total column occurred in September, challenging most of the existing ozone depletion hypotheses<sup>5-8</sup>, which can more readily account for a change in October. The total ozone levels

in October were affected by substantial increases in ozone near the 24–30-km level, which were associated with planetary wave activity at these upper levels. Year-to-year variations in the October total column abundance may reflect such variations in upper level dynamics even as lower altitudes suffer further depletion.

Also important is the observation<sup>2</sup> of restricted layers in which dramatic depletions of the local ozone abundance had occurred, making important contributions to the integrated column change. The layers were typically a few kilometres in thickness, and many structures repeated on consecutive soundings, demonstrating that they are not an instrumental artefact. On one occasion in October a local depletion of more than 90 per cent was observed in a layer a few kilometres thick at 60 mbar (note that the observed depletion of the integrated column on this date was about 50 per cent). Aerosol measurements on the same flight showed no evidence of corresponding layers that would probably be associated with a dynamical explanation of this phenomenon. On the other hand, previous chemical hypotheses proposed to date do not suggest such large, local ozone depletions. Hofmann *et al.* suggest<sup>2</sup> that the layered structure is associated with chemical reactions following the evaporation of polar stratospheric clouds. These observations may represent the greatest clue and challenge to the mystery of the cause of the ozone hole.

It seems clear that much further research is needed to clarify the cause of this remarkable phenomenon. The Antarctic temperature climatology is of prime importance. Further observations of chemical species, in addition to nitrogen dioxide and ozone, are badly needed, both from the ground and *in situ*. Dynamical tracers such as nitrous oxide and methane should be measured, and laboratory studies of heterogeneous reactions and chlorine chemistry carried out. *In situ* observations of a range of chemical species inside one of the observed layers of spectacular local depletion would undoubtedly add much to the understanding of their origin. □

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## Imanishi's influence on evolution theory in Japan

SIR—My Commentary<sup>1</sup> on Imanishi's theory of evolution and the Japanese dimension evoked a gratifying range of responses<sup>2-9</sup>. Several correspondents<sup>3,4</sup> complained about my article being more sociological than scientific. This is indeed the case as my aim was primarily to describe Imanishi's theory and set it in its social context — there is little to be said about its scientific content.

Whereas Nakahara *et al.*<sup>5</sup> claimed that I had rejected Imanishi's theory without fully understanding it, the actual wording of my summary was checked by Imanishi himself who stated "I was very impressed that you have got the ideas very precisely in spite of the language difficulty".

Some of the Western correspondents, such as Sinclair<sup>3</sup>, obviously found Imanishi's theory so unusual that they could not believe what I had carefully written. Sinclair states "Imanishi no doubt would not deny that interspecific competition exists", yet that is precisely what Imanishi contends; such denial is the very essence of his theory and it is this which makes it unique. I certainly did not confuse the roles of intraspecific and interspecific competition. Needless to say, I agree that natural selection and the entire edifice of Darwinism requires intraspecific competition. Similarly, I agree with Millar *et al.*<sup>4</sup> that aspects of Imanishi's theory are not unique, such as the denial of the importance of intraspecific competition and his assertion "that the unit for explaining evolution is not the individual but the species". Many of Imanishi's ideas are mirrored in the separate and joint writings of Eldredge and Gould<sup>10-13</sup>. Indeed, Imanishi has intimated his sympathy with much of Gould's writings, and is especially sympathetic to the notion of sudden jumps in evolution, and states "evolution is change occurring when it is time to change".

It is perhaps significant that these self-same ideas are to be found in the writings of Lysenko<sup>14</sup> (with his own emphasis):

"No continuous, unbroken series of forms between species — different qualitatively definite states of living matter — have been found. This is so not because the intermediate forms in a continuous range have died out as a result of mutual competition, but because there is no such continuity in nature, nor can there be. A species is a distinct, qualitatively definite state of living matter. We must realize that speciation is a transition — in the course of the historical process — from quantitative to qualitative variations. Such a leap is prepared by the vital activity of organic forms themselves, as the result of quantitative accumulation of responses to the action of definite conditions of life, and that is something that can definitely be studied and directed. The conversion of one species into another takes place by a leap."

Interestingly, Japan is one of the few places where Lysenko is still taken seriously. The Japanese Society for Michurin Biology was set up in 1954, the *Japanese Journal of Michurin Biology* was launched in 1965 and still continues in the 1980s. There is clearly a sympathetic response to these ideas in Japan.

My brief discussion on the scientific basis of Imanishi's concepts of proto-identity and habitat segregation or life style partitioning, which I claimed had been effectively refuted, was challenged by Sibatani<sup>2</sup> and Millar *et al.*<sup>4</sup>. The points they raised have been adequately dealt with by Rossiter<sup>6</sup>. I agree that habitat segregation is a real phenomenon, as Nakahara *et al.*<sup>5</sup> insist, but it is not as they or Imanishi suppose antithetical to natural selection. The late Kani and Imanishi's close colleague Morisita<sup>15</sup> recognized the importance of interspecific competition. There is no conflict regarding observations of the phenomena. It is in the explanations proposed to account for them that disagreements arise. Hence I agree completely with Sakura *et al.*<sup>8</sup> that "Imanishi's description of phenomena is not incompatible with Darwinian theory which deals with mechanisms of evolution".

Finally, I was pleased to read Sibatani's<sup>2</sup> comments on "Japanese ethnocentric excesses" as well as the letter from the primatologists in Kyoto who say that although they "are still hampered by the influence of Imanishiism, we criticize his theory and try to eliminate its negative influence"<sup>18</sup>. This contrasts with Asquith<sup>9</sup> who challenged me on my general comments on the average intelligent layman's understanding of evolution, which I do not retract.

Darwin's work was indeed introduced into Japan in the last century and in fact social darwinism was one of the cornerstones of the social policy of the rigid authoritarianism of the Meiji restoration. It is against this particular background that the importance of Imanishi's opposition to Darwinism must be viewed.

Imanishi is still a national hero, as he was in the 1930s. The popular press abounds with interviews and articles by and about him. Typical is the article in the English language magazine *Look Japan* (10 January, 1980) "Kinji Imanishi, Japanese Theorist — as compared to Darwin", in which the three great thinkers of modern times are seen as Darwin, Marx and Imanishi. The interview with Imanishi in the July 1984 issue of *Omni* (Japanese edition only) gives a very clear idea of the high esteem in which he is held.

Asquith<sup>9</sup> confirms my critique of Japanese scientists. According to her "the majority of Japanese scientists simply disagree with Imanishi's popularizations and find his views obscure and untestable. Their silence is hardly surprising". My

account was an attempt to draw attention to Imanishi's theories and to set them in their context, and if possible to provoke the Japanese scientific community into some kind of response. Sibatani's<sup>16</sup> initial aim was to bring Imanishi's ideas to the attention of Western scientists. I was the first to respond. I set out in my book<sup>17</sup> to bring to the attention of Japanese scientists the considered opinion of an individual Western scientist on the theories of Imanishi. I believe that both Sibatani and I have succeeded in our avowed intentions.

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● This correspondence is now closed — Editor, *Nature*.

## Macrophage regulation of vitamin D<sub>3</sub> metabolites

SIR—In his provocative News and Views summary<sup>1</sup> of some features of the vitamin D system, Ian Dickson discusses the formation of the active 1,25-dihydroxyvitamin D<sub>3</sub> metabolite from 25-hydroxyvitamin D<sub>3</sub> by the enzyme 1-hydroxylase in the kidney. He also refers briefly to the existence of receptors for this metabolite on macrophages and in the thymus. The considerable importance of the latter observations is more obvious when one realises that macrophages can also express the 1-hydroxylase, which had been thought to occur only in the kidney. Moreover, this activity is enhanced dramatically when macrophages are exposed to gamma interferon<sup>2-4</sup>.

Thus the 1,25-dihydroxy metabolite formed by the macrophages can have an autocrine effect on these cells. For instance, in the system we have studied it increases the ability of cultured human monocytes to inhibit the *in vitro* growth of

*Mycobacterium tuberculosis*<sup>4</sup>, possibly explaining the well documented reports that vitamin D therapy cures chronic skin tuberculosis<sup>5</sup>. Because T lymphocytes also contain the receptor, presumably they are also affected by the metabolite, but with what functional significance is not clear<sup>6</sup>.

Thus vitamin D<sub>3</sub> metabolites have immunologically relevant effects on the lymphoid system, and their levels within this system are locally regulated by T cells, gamma interferon and macrophages, in a manner which may usually be quite independent of the regulation of systemic levels. When interferon-induced macrophage activation is intense, however, 'overspill' of the 1,25-dihydroxy metabolite can occur, causing sufficient rises in systemic levels to induce hypercalcaemia, as often seen in sarcoidosis<sup>2</sup>, and sometimes in tuberculosis<sup>7</sup>.

Once again the macrophage proves to be a cell with an amazing range of potential functions.

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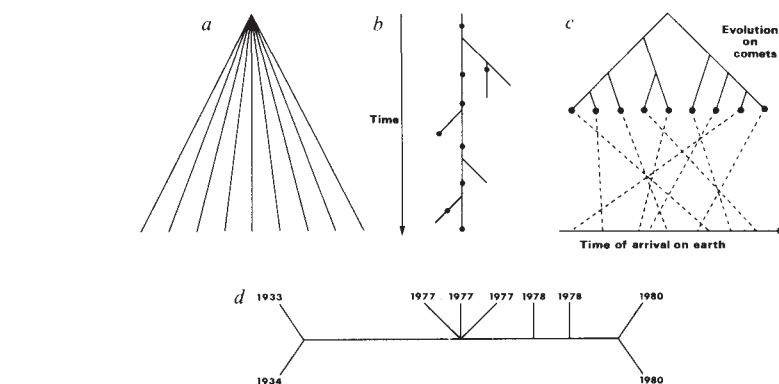
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## Models for the origin of influenza viruses

SIR—Hoyle and Wickramasinghe have recently repeated<sup>1,2</sup> their hypothesis<sup>3</sup> that the Earth is continually bombarded by influenza viruses from comets. Their 'comet' model denies transmission of influenza viruses between individuals, each infection being contracted directly from virus particles falling through the upper atmosphere. The time is past for such qualitative speculations now that the predictions from the comet model can be falsified by quantitative tests.

The predictions concern the minimal-length trees that can be constructed on the basis of nucleic-acid sequences. The largest set of sequences for one strain of influenza virus from the same host are nine haemagglutinin sequences covering the years 1933 to 1980 of the human H1 subtype. The simplest comet model is that the different isolates of H1 influenza are carried on different comets, having developed independently from a common ancestor that existed at some distant time in the past. On this model the sequences would appear generally similar because of their common origin (thus explaining their homology), but the differences between



**Fig. 1** Trees representing different models. *a*, The Big-Bang or star tree model. *b*, The biological model (for example ref. 7). *c*, Modified comet model (independent arrival). *d*, The minimal tree<sup>8</sup> for the nine viral sequences from the EMBL database<sup>9</sup>, edges of zero length are contracted.

different pairs of taxa would be independent. This is the Big-Bang model<sup>4</sup>, and is represented by the star tree<sup>4,5</sup> shown in Fig. 1*a*. If, for example, three viral isolates had adenine at one nucleotide position and the other six had cytosine then the star tree would require three nucleotide changes while the biological model, which assumes that viral strains share common ancestors (Fig. 1*b*), could require one, two or three changes. The expected number of nucleotide changes for a minimal-length tree, given the Big-Bang model, can be calculated (unpublished data). For these nine sequences, the expected number is 163 whereas the observed number is 95. This has a probability of occurring of about  $10^{-58}$ , assuming that the viruses arose independently from a common ancestor. Thus we reject the star tree model.

The observed number of changes is however consistent with an evolutionary model whether it be terrestrial or extraterrestrial. For example the second version of the comet model (Fig. 1*c*) assumes that viruses on different comets are related through some ancient extraterrestrial evolutionary process but that they arrive independently on earth from different comets. Viruses on two comets may share a more recent ancestor than with those on a third comet but any relationships among the viruses should show no significant correlation with their order of arrival on earth. This leads to the prediction that influenza viruses isolated in consecutive years should not be more closely related than those isolated several years apart. In fact, the viruses on the minimal tree (Fig. 1*d*) are in chronological sequence, consistent with the biological model. There is one chance in 7,560 ( $=9!/8.6.2$ ) of obtaining this time sequence if the viruses on the minimal tree arrived independently in time. Extending the data set to 12 viral isolates (1933-1982) for which a smaller part (312 nucleotides) of the haemagglutinin gene is sequenced, leads to an even smaller probability. Twelve taxa with independent arrival times, will produce a

chronological order as good as that observed (one pair of adjacent isolates interchanged) about once in 1,500,000 trials. Consequently we reject this second comet model.

Models invoking only a single comet do not fit the data either. One such possibility is that of a single comet carrying an evolving virus that periodically infects the Earth as the comet passes it. But the pattern in the arrival of virus isolates, the short times between altered sequences, the lack of a slow mixing compartment in the atmosphere and the lack of randomization of sequences to give a 'quasi-species'<sup>6</sup> lead us to conclude that this single-comet hypothesis cannot explain the evolution of influenza viruses while still retaining the assumption of non-transmission between host individuals.

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## Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of less than 500 words and five references. □



# A drive with Dr Klein

Jonathan Howard

**Natural History of the Major Histocompatibility Complex.** By Jan Klein.  
Wiley: 1986. Pp.775. \$95, £90.75.

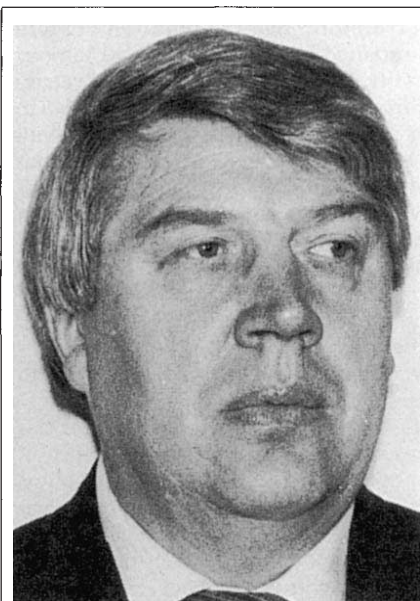
JAN KLEIN admits outright that his enormous new book does not easily fall into any of the established categories of textbook, monograph or review. But perhaps the more interesting question for the critic is not what the book is but why it was written. Many and powerful impulses must have driven Klein to produce a volume of such size and scope, an astonishing labour for one busy man, but the account of them in the preface does not make wholly reassuring reading.

First, Klein rejects what he feels is an aesthetically and morally disreputable view that no single person can come to grips with a whole field; "the philosophy of the assembly line.... I have always thought that if I were to contribute to the construction of a car, I would want to drive it as well". The anxious reader might here wonder whether an assembly line worker is necessarily the best driver. Secondly, there was an impulse not just to come to grips with the whole field, but actually to *contain* it. With the example of Buffon's 36-volume *Histoire Naturelle*, Klein justifies both his slightly odd title and the attempt to make this account of immunogenetics not merely inclusive in its own field but inclusive of everything. If our first fear was that the drive might be unsafe, our second is that we might well get lost.

Klein's third stated impulse to write this book is the most unsettling of all: "I also hope to have achieved one further goal: that of discouraging certain lines of research". There are, perhaps, some innocuous readings of this extraordinary declaration, but a glance at Table 7.42, to which Klein refers the reader in this context, makes it clear that they do not apply. There are not many fundamental problems left in immunology, but one that would be on anybody's list is the subject of this table, namely the relationship between T-cell function (that is, help, cytotoxicity and suppression), the specificity of the T-cell receptor for class I or class II MHC molecules, and the expression of the co-receptor molecules, CD4 and CD8 or their homologues. To our other fears, therefore, we must add that the driver seems to have taken an unreasonable dislike to the opinions of most of his passengers.

It must be admitted that these fears are not groundless. Klein's great book, *Biology of the Mouse Histocompatibility-2 Complex*, was subtitled *Principles of Immunogenetics Applied to a Single System*.

Ten years later the boot is on the other foot, and it is the MHC whose principles apply to many systems. The MHC is a peculiar object of study. Functionally a part of the mechanism driving the immune response, to which uniquely, as I and Klein both believe, it owes its properties, it now also provides an object lesson in the study of balanced polymorphism, multi-gene families, ecological and evolutionary genetics, genetic mechanisms such as



Jan Klein — a man of conviction.

mutation, gene conversion and recombination, developmental regulation and much more. Klein's instinct that this topic contains something of value for every biologist is surely correct. However the relevance of MHC studies to so many disciplines puts the would-be expositor into a peculiarly difficult position. There is no real 'field' of the MHC for Klein or anyone else to come to grips with. For example, immunology is a highly-structured discipline in which the MHC-specified glycoproteins play a central role, but the genetic complexity at the chromosomal and population levels, which provides much of the MHC's renown, is, in the context of immunology, often a convenience, usually an irrelevance and occasionally a damn nuisance. In other disciplines it is the genetic properties of the MHC that matter, while the immunological activity of this or that product is beside the point. In the case of human tissue typing it is the sheer diversity that matters, with

all the impenetrable nomenclature that follows.

With the most extraordinary courage, Klein has taken on the whole thing. Following an approach that he pioneered in his 1982 textbook, *Immunology: The Science of Self-Nonself Discrimination*, he tells us not only everything that he knew before he started work on the book, but also everything that he found out after he got going. As a result, what was already certain to be a mighty production seems almost to have burst the bounds of reason. Where else can you find a detailed account of DNA sequencing by limited chemical cleavage but no mention of dideoxy chain termination? A weird mnemonic for the single-letter amino acid symbols (Tryptophan = W? tWo rings, of course)? A picture of *Lens culinaris* from which lentil lectin is purified? Gametogenesis and sexual morphology in flowers? The life-cycle of bacteriophage lambda? And so on and on. Klein-Diderot more than Klein-Buffon.

One would, perhaps, be better prepared for this torrent of information if Klein were not so dreadfully strict about other matters. For Klein, the MHC is precisely and exclusively "that group of genes coding for molecules that provide the context for the recognition of foreign antigens by T lymphocytes". No complement genes, no steroid hydroxylases, no neuraminidases, none of the things that one might vaguely include in the MHC by virtue of the fact that they are usually all bundled up with Klein's genes on the same chromosome, even if one does not understand why. On the other hand, Klein points out that his definition allows him to include  $\beta_2$ -microglobulin even though it maps to a different chromosome. In the event one need not worry too much about possible exclusions, because, in the encyclopaedic spirit, there is in fact a chapter devoted to them, including among an enormous number of other things a picture of the whole complement system, much of which is nothing to do with the MHC by anyone's definition.

Such a book makes exceptional demands on the index; sadly, that provided is not up to the task. Anyone who wished, for example, to track down the passage on page 137 where Klein describes the evidence that at last convinced him that unidirectional genetic exchange was responsible for much of the peculiar sequence variation in MHC genes will not, surprisingly, find it under either *Gene Conversion* or even *Conversion*, even though at least the latter would seem entirely appropriate.

The sheer prodigality of the information carried in this book, whether in the text, the figures or the wonderful tables which enshrine the published history of every MHC-related topic, make it quite

unique. But there is a price to pay, and I do not mean £90.75. This is a book of opinion and judgement as well as fact, and while Klein's views are often balanced and helpful, as in the excellent discussion of the *MLs* locus problem, they are often capricious and sometimes plain offensive, as in the repeated insinuation that his fellow scientists have obtained results in bad faith. When the topic is centrally important to immunology, this high-handed treatment is a serious defect. For instance, the handling of antigen before it is presented to specific lymphocyte receptors is crucially important, and, unlike a lot of material in the book, is strictly relevant to the properties of MHC molecules as well. Unfortunately it is also one of the topics where Klein feels that most immunologists are wrong-headed. That is to say, not only does he feel that he is right, but also that it is a topic which is best presented in such a way that some hypothetical objective reader can appreciate how silly most immunologists are. The trouble with this approach, apart from the unpleasant sensations that it excites in someone who might have inclined to the majority view, is that it fails to prepare the reader for the proper appreciation of work published after Klein's book was completed. It is especially sad in this case. Last year it was shown that membrane class I and class II MHC molecules probably acquire processed antigen from different intracellular pools. If generally true, this finding has tremendous implications for the future development of immunology, especially the immunology of virus infection, as well as making a dramatic and so far unassimilated contribution to cell biology. It is also giving most immunologists a great deal of enjoyment. An authoritative teacher who ridicules an important argument that turns out to be right has a lot to answer for.

But it is always a pleasure to read Jan Klein, even when one disagrees with what he says or disapproves of how he says it. He is a fluent writer with an enviable ability to say clearly what he means. His writing is, in that sense, transparent, so that one seems to see the man himself speaking through the page. He has enormous convictions, and it is difficult not to fall foul of at least some of them. But Klein has enormous courage too, an important virtue and one that demands an equal and often opposite courage in his audience. Do not necessarily believe what Klein says; take the trouble to find out your own point of view for yourself. If it conflicts with Klein, then take issue with him; he may well be wrong. If he forces you to think again about why you believe that something is so, even if you end up with your own conviction strengthened, then he has done something of rare value. □

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## Who dunnit down at the gravel pit?

Michael Day

**The Piltdown Inquest.** By Charles Blinderman. *Prometheus*: 1986. Pp.261. \$22.95.

IT is a strange facet of humanity that, as a species, we are intensely concerned with transgressions of our own codes of behaviour. Distinguished academics write whodunnits, television programmes are obsessed with crime and its detection, successful art forgers such as Van Meergeren and Tom Keating are folk heroes, and even the Great Train Robbery is part of modern British history. It is not surprising, therefore, that the Piltdown Forgery has retained its place as the most famous scientific fraud of this century. The recovery from a Sussex Pliocene gravel pit in 1908 of a piece of 'human' fossil skull began a series of so-called discoveries that culminated in the reconstruction of Piltdown Man from what turned out to be an orang-utan jaw and other fossilized skull bones. The skull was believed by many to represent an example of the 'missing link' between ape and man that seemed to be needed to give substance to Darwin's view of the origin of species as applied to man.

It was not until 1953 that the fraud was exposed by the use of the modern techniques of the day, including fluorine analysis of bone, analysis of the paint on the specimens and the detection of radioactivity in some hippopotamus teeth in the pit that gave away their provenance. The forgery was exposed in 1953, but not the forger. Over the past 30 years, almost everybody who was in any way connected with the discovery or examination of the remains has been a suspect, and many books and articles have been devoted to proving that one or other famous figure was the perpetrator. Blinderman's book, the latest in the line, is a timely review conducted in the form of a historical inquest; it is well researched, accurate and readable, even if its style is racy on occasion. Blinderman writes with assurance, if not authority, evidently gained from an exhaustive

• Just published in the United States (by Houghton Mifflin) is *The Red Ape: Orang-utans & Human Origins*, by Jeffrey H. Schwartz. The book is tendentious, the author examining the "often unacknowledged biases and preconceptions" of palaeoanthropologists and aiming to show that the closest relatives of *Homo sapiens* are not the African apes but the orang-utan.

Publisher in Britain will be Elm Tree, an imprint of Hamish Hamilton, and the book will be reviewed in a future issue of *Nature*.

search of the literature and other extensive inquiries.

Following the story of the discovery and the exposure of the forgery, Blinderman offers a profile of the hoaxer; then the ten main suspects are examined one by one and the field narrowed down. The line-up of the culprit (or culprits, for conspiracy theory is not ruled out) includes, alphabetically, Lewis Abbott, William Butterfield, Charles Dawson, Arthur Conan Doyle, John Hewitt, Martin Hinton, Grafton Elliot Smith, W.J. Sollas, P. Teilhard de Chardin and Samuel Woodhead. Clearly some of these are more suspect than others. Foremost amongst them, of course, is Charles Dawson, the local solicitor and scientific savant who 'found' many of the pieces and who is the culprit favoured by J.S. Weiner, one of the prime movers in the uncovering of the hoax. It is true, however, that Weiner was persuaded to name Dawson publicly only when pressed by Lord Zuckerman in 1972 after accusations directed at Grafton Elliot Smith had distressed Smith's family.

Perhaps the only piece that I can add to the jigsaw is that Weiner must have the credit for realizing that the explanation for the strange anatomy and the fluorine results had to be fraud. Weiner told me that after visiting the British Museum and examining the Piltdown remains yet again, he drove home to Oxford one evening. While musing on the day, he realized that the lack of co-planarity in the wear pattern of the Piltdown molars could have been produced only by artificial means. The teeth must have been tampered with after death. As with every conjuror, you fail to understand the trick because you never believe that he would be so mean.

After that flash of insight, the other features fell into place; the broken condyle, the brown paint and the jaw's high nitrogen content. The ape-like anatomy of some parts of the jaw was also explained — simply, it had belonged to an ape.

There is no doubt that the Piltdown forger was knowledgeable, skilful, cunning and fortunate. The gullibility of the scientists at the time seems likely to have been due not to stupidity but to a desire to see evidence for a theory that was so compelling and satisfying. The hoaxer could lead them on from find to find, confirming their best guesses every time and feeding their scientific vanity. We are never so vulnerable as when others are confirming our views.

Not all of the scientists of the day were fooled, however. As early as 1913, the British anatomist David Waterston refused to accept that the jaw and the skull had come from one individual, claiming that X-rays proved that the jaw was that of an ape. In the United States, Gerrit Miller took the same view, as did a number of continental authorities. The doubts faded as the forger/hoaxer salted his mines and



more and more material came to light. Was it really a hoax that went too far to be admitted? Was it an attempt to make a fool of a rival? Was it a serious but fraudulent attempt to 'prove' Darwin's theory? The question of motive is just as intriguing as the identity of the forger.

Blinderman's inquiries range widely, even entering the realm of fantasy when Conan Doyle is considered suspect and Sherlock Holmes is brought in to help. Does he succeed? Which of the ten suspects is finally convicted? You'll have to read the book to find out. □

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## Energetic exercise

Ian Smart

**Energy 2000: A Reference Projection and Alternative Outlooks for the European Community and the World to the Year 2000.** By Jean-François Guilmot, David McGlue, Pierre Valette and Christian Waeterloos. *Cambridge University Press: 1986. Pp.261. £25, \$44.50.*

GIVEN the circumstances of the past 15 years, it takes courage to publish a projection of energy demand and supply. And only the brave would ever reveal the results of an exercise in formal modelling. What is ostensibly a dry discourse is thus doubly valorous.

Building on research promoted by the European Commission over a ten-year period, what we have are accurately described by the sponsors as "consistent energy projections on a country basis and at the level of the Community as a whole, using throughout the same methodological approach and harmonised energy data and balances" (p. viii). The methods are conventional, entailing the choice of a single socio-economic scenario for the latter-day Community of Ten (that is, excluding Spain and Portugal) and using that scenario to establish a reference projection of energy demand and supply until the end of the century, surrounded by less detailed 'high' and 'low' variants. What is less usual and more stimulating is that aggregated results for the Community have been assembled from a series of standardized and consistent sub-projections of national energy markets.

The result is a workmanlike job. Readers drawn by an interest in modelling will regret that there is little specific information about the algorithms employed. Those looking for a reference work will wish that, in the absence of an index, a detailed catalogue of contents and tables could at least have been included. And no

one is likely to cherish the text as light reading. Yet the exercise would still have justified itself had it done no more than coax and cudgel evidence from ten different energy economies into a common analytical frame.

In fact, the study attempts more — but not always so successfully. It necessarily has a good deal to say about energy costs and prices, for example. But the handling of how input costs are translated into consumer prices, or prices related to investment propensity, is too tentative and opaque to be convincing. That may be because the Commission is sensitive to how Community members will feel about a study of this sort. Repeating that the authors' energy projection is not a forecast but only "a plausible and consistent scenario" cannot conceal that it reflects what they take to be the probable evolution of policy as much as of economic circumstance. Indeed, their methods included consulting the views of national governments, and the effect is sometimes apparent. Even in 1985, for instance, few analysts would have wagered on Italy commissioning another ten nuclear power reactors before the end of the century, or Britain building not only Sizewell B by the year 2000 but six more similar units as well.

As it is, the plausibility of what is projected here was largely demolished in 1986 by the collapse of oil prices and the disaster at Chernobyl. Ironically, that is almost to the report's advantage, because it removes the temptation to consider it as anything but a valuable effort to make energy systems analysis in ten different countries more rigorous, transparent and consistent. But the extraordinary history of 1986 also throws into relief the study's most fundamental weakness, which lies in its treatment of uncertainty. Many inputs to the model are properly described as uncertain. The output projections, however, still seem to assume that policymakers will be confidently rational in choosing an optimal response for the long term. As a result, there is no allowance for how traumatic experiences undermine their confidence, and increase the pressure to reject long-term commitments in favour of more easily mutable short-term palliatives. They, too, are uncertain.

This report is testimony to a brave beginning. As good modelling studies should, it purports to give no answers, but only to help in asking progressively better questions. The European Commission should therefore be encouraged to repeat the exercise. If it does, however, it will need to take account not only of economic uncertainty in energy matters but also of nervous managers and frightened politicians. □

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## Star-like qualities

Malcolm G. Smith

**Quasar Astronomy.** By Daniel W. Weedman. *Cambridge University Press: 1986. Pp. 217. £25, \$39.50.*

*Quasar Astronomy* is an excellent observers' research guide to the violent universe of quasars and active galactic nuclei. Daniel Weedman has been observing Seyfert galaxies and quasars at a wide variety of wavelengths, using a wide variety of techniques, for the past 20 years. His teaching credentials in this field are equally impressive. As he reminds us in his preface:

I already owed a debt to the late Carl Seyfert, who had given me my first encouragement in astronomy when I was a high-school student... this book is a product of my attempts to explain [quasar astronomy] to students in various classes at the University of Texas, Vanderbilt University, University of Minnesota and Pennsylvania State University.

From the outset, Weedman makes it clear that technology has played an essential role in the study of these very distant sources of energy. He writes:

To be honest... most of the progress [in understanding quasars] should be credited to the engineers and physicists who have developed tools that allow our wide-ranging probes into the mysteries of quasars.

That point of view may be a little extreme, and could be contested by a few of the eminent theoreticians who have worked in this field, but the quotation serves to illustrate some of the flavour of Weedman's approach.

This is not a difficult book. Weedman's account is basic and straightforward, and is illustrated with simple worked examples. He takes the view that the complications stemming from the maze of unit conversions and cosmological formulae are important enough that most students of the subject would benefit from having the details explained and set out explicitly. He remembers that most of us took a number of years to become reasonably comfortable with all the practical corrections and conversions — and that most of us worked through many of the derivations essentially from scratch, and then kept the results in a notebook to save having to do them all again!

Weedman's treatment of the physics of quasars is highly simplified. Yet it shows the insight that his deceptively simple observational approach to research has brought to this field, and the book will be of great value to those embarking on the study of quasar astronomy. □

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# Changing physics

Cyril Domb

**James Clerk Maxwell and the Theory of the Electromagnetic Field.** By John Hendry. *Adam Hilger: 1986. Pp.305. £30.\**  
**Maxwell on Molecules and Gases.** Edited by Elizabeth Garber, Stephen G. Brush and C.W. F. Everitt. *MIT Press: 1986. Pp.565. \$55, £54.95.*

WHEN the Cavendish chair of physics was established in 1871, it was offered first to Sir William Thomson (later Lord Kelvin) and then to Helmholtz. Only when both of these had declined was James Clerk Maxwell approached. Several years had passed since the publication of Maxwell's classic papers on the electromagnetic field, but their significance was far from being appreciated. As late as 1884 Thomson wrote in the preface to his Baltimore Lectures that "the so-called electromagnetic theory of light has not helped us hitherto". Maxwell's contemporaries regarded him as a top scientist, but not as one of the great innovators of history.

The first decades of this century saw the development and exploitation of radio waves, and the formulation of the special theory of relativity and of Planck's quantum hypothesis, in all of which Maxwell's electromagnetic theory played a central role. The physicist of the twentieth century has no doubts that Maxwell deserves to rank with Newton and Einstein. In a celebration of the centenary of Maxwell's birth in 1931, Einstein described the change in conceptions of physical reality after Maxwell as "the most fruitful that physics has experienced since the time of Newton". In addition to his epoch-making ideas on electromagnetic theory, Maxwell made outstanding contributions to statistical mechanics, kinetic theory, colour vision and a host of other topics, and all of this in a short life-span of 48 years. But although Newton and Einstein are household names, the average man in the street has not heard of Maxwell.

During the past few years efforts have been made to put Maxwell on the map where he belongs. The excellent short biography by Everitt in 1974 contained interesting new material (Maxwell died in 1879 and it was still possible to make use of living testimony). More recently, the centenary of Maxwell's death stimulated new biographies by Tolstoy in 1981 and Goldman in 1983.

The books under review are welcome additions to the literature on Maxwell, and are designed for a more professional audience of historians and philosophers of science. They deal with different aspects of Maxwell's work, Hendry being con-

cerned with electromagnetic theory, and Garber and her colleagues with statistical physics and kinetic theory. The approaches also differ. Hendry has not made much attempt to unearth new archival material, and has relied on what is already available in the literature, whereas Garber *et al.* reproduce Maxwell's publications and letters in a systematic manner with a learned commentary, one of their aims being to make widely available material which has previously been known only to specialists.

Hendry starts with a comprehensive account of the historical development of electricity, magnetism and electromagnetism at the beginning of the nineteenth century, tracing the contributions of Oersted, Ampère, Faraday and Thomson. This sets the stage for Maxwell's entry. Maxwell was deeply influenced by Faraday's idea of a field filled by lines of force, and his initial aim was to give mathematical expression to this concept. In fact his preliminary paper, written in 1855 while he was still a student at Cambridge, was entitled "On Faraday's Lines of Force". The first two of his classic papers on the electromagnetic field, published in 1861 and 1862 under the title "On Physical Lines of Force", made extensive use of molecular vortices to explain lines of force and electric currents; in the second paper he introduced a mechanical analogue, and this led him to postulate a displacement current. The mathematical consequence of this postulate was the existence of electromagnetic waves with a velocity determined by electric and magnetic quantities. When numerical values were inserted the velocity turned out to be close to that of light. In his final paper, "A Dynamical Theory of the Electromagnetic Field", published in 1865, the superstructure of molecular vortices and mechanical analogues was swept away, and the equations were put forward in their superb symmetric form.

Hendry supports the view of T.S. Kuhn that discovery is rarely a simple event and that it should be treated as a process. He emphasizes that Maxwell's great contribution was the understanding he provided of the nature of electromagnetism. The identity of the velocity of electromagnetic and luminiferous waves was just one point in an elegant and sophisticated structure. Others (including the distinguished mathematician B. Riemann) had suggested this one point, but had been unable to provide an acceptable physical framework.

A good deal of space is devoted to an analysis of the difference between the mechanistic and dynamistic approaches in the history and philosophy of science. I did not find the discussion very relevant, but this may be due to my training as a physicist. One feature caused me some irritation. An important advantage of the sys-

tem of referencing by dates is the historical perspective gained when one reads the text. This is lost if the reference is not to the original publication. A reference to J.C. Maxwell (1982) is bizarre; it is much more appropriate to write J.C. Maxwell (1865) and in the reference list add 'reprinted 1982...'.  
 In the second volume under review, the published papers and manuscript writings

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*Maxwell — epoch-making ideas.*

are handled in a highly professional manner. Both the preface and the introductory article entitled "Kinetic Theory and the Properties of Gases: Maxwell's Work in the 19th Century Context" are useful and informative. Notes on the archival material save the reader from puzzling over unfamiliar phrases in the letters and postcards.

The published papers are drawn largely from the two volumes of Maxwell's collected works edited by W.D. Niven (reprinted by Dover in 1965, though now alas again out of print). But some material which slipped through Niven's net is included here, for example an exchange of letters in *Nature* with A.J. Mott on "Atoms and Ether", and an *Encyclopaedia Britannica* article on "Physical Sciences" published posthumously.

A major source of letters and articles is the classic biography of Maxwell by Campbell and Garnett, published within a few years of Maxwell's death and happily available again as a Johnson reprint. But the most novel features of the book are the letters and manuscripts reproduced from private collections, most of them coming from the Maxwell collection in the Cambridge University Library. Other letters have already appeared in print but are scattered through, for example, biographies of Stokes and Kelvin.

Both volumes provide ample evidence of the care which Maxwell devoted to every statement he made; of his coherent and logical thinking; and of his magnificent command of the English language and unique personal style. □

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# Programming a highly parallel computer

W. Daniel Hillis and Joshua Barnes

*Parallel computers potentially offer rates of computation far above those of conventional machines but using them efficiently requires understanding of their capabilities and limitations. Algorithms that simulate the N-body problem on an existing parallel computer with 65,536 processors provide an illustration*

A NEW type of computing engine, the parallel computer, offers opportunities to perform scientific computation that have previously been impossible. Unlike conventional computers that perform one operation at a time, parallel computers can perform tens of thousands of operations simultaneously, making them very much faster. But high performance cannot be achieved for all algorithms. Just as the electron microscope offers a far higher magnification than the optical microscope, without replacing it, the parallel computer offers much greater rates of computation for certain problems, but it is not a direct replacement for conventional sequential machines. Here we shall review some of the capabilities of a 'fine-grained' parallel computer, one that contains a large number of computational elements and is particularly suited for the study of systems containing a large number of interacting elements.

The algorithms described are 'data parallel' rather than 'control parallel' in that they can perform simultaneously atomic operations on large sets of data. For example, adding two large vectors containing many components is an atomic operation on a parallel computer in the same sense that adding two numbers is an atomic operation on a conventional computer. This type of data parallelism may be contrasted to control parallelism, where concurrency is achieved by coordinating the activities of many programs simultaneously. The data-parallel style of programming is a natural one when tackling computations involving large numbers of similar elements, as are often found in scientific problems. In the example that we discuss, the discrete elements are the positions and velocities of individual stars and the gravitational forces between them. The computational problem is to simulate the motion of the stars over time.

In reality, all stars move simultaneously, so it is natural to model the system by an algorithm in which all elements of the data structure are computed simultaneously. In the execution of the algorithm, an individual computer processor is attached to each element of data, in this case the position or velocity of a single star or the force between two stars. The algo-

ithm is executed in all the processors at once, and hence for all the stars at once. Each step of the algorithm operates on a large set of data elements. In the algorithms we describe, time in the execution of the algorithm is directly analogous to time in the modelled system, although this is not a necessary feature of a data-parallel algorithm.

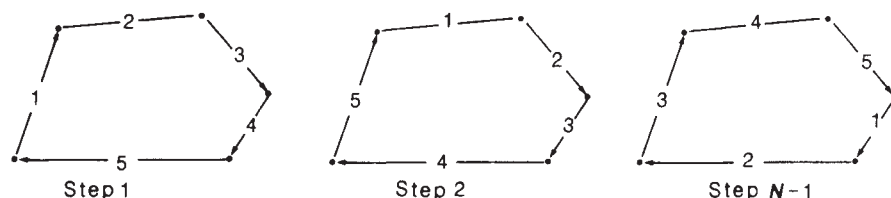
## Parallel architecture

The structure of conventional algorithms reflects the word-at-a-time structure of conventional computers. In the same way, data parallel algorithms reflect the structure of those parallel computers that contain tens of thousands of relatively simple processors. Such machines are referred to as 'fine-grained' parallel computers, in contrast to 'coarse-grained' computers, which contain up to a few hundred relatively conventional processors. As an example of a fine-grained parallel computer designed to be programmed in the data parallel style, we shall describe the system called the Connection Machine<sup>1</sup> (Thinking Machines Corporation) a commercial parallel machine containing 65,536 processors, each with its own memory. Other examples of fine-grained computers are the Massively Parallel Processor<sup>2</sup> (Goodyear Aerospace) and the Distributed Array Processor<sup>3</sup> (ICL).

The Connection Machine has three major components: the processor/memory cells, a conventional front-end computer and a communication network that allows the processor/memory cells to exchange information in arbitrary patterns. The front-end computer is a standard sequential scientific computer (such as DEC's VAX computer). The user programs the computer from the front end, using simple extensions of conventional high-level language, with conventional control structures.

The Connection Machine processor/memory cells connect to the front-end computer as if they were a large conventional memory. In its simplest mode of operation, the processor/memory cells of the system can act simply as memory, supporting read-write operations of the front-end machine. Data stored in this section of the memory can be operated upon in the standard word-at-a-time manner, but it is also possible to perform operations on all of the data at once. For example, all stars can simultaneously update their positions according to their velocities. The program sequence that determines the algorithm is stored once, in the front end. Connection Machine hardware broadcasts each command to all processors, so that each can perform a local operation on its own unique data. The time required to perform elementary operations depends on the size of the data field. For example, operating in parallel, a Connection Machine system is able to do  $8 \times 10^9$  8-bit additions, or  $1 \times 10^9$  64-bit additions, per second.

Many important operations require communications between processors. Communication is necessary, for example, to compute the gravitational interaction of the stars. Fine-grained computer systems differ in the type of hardware mechanisms that are provided for in such communication. In the Distributed Array Processor and Massively Parallel Processor systems, the processors are wired in a two-dimensional grid. In the Connection Machine system the processors are interconnected by a switching network that allows any processor to communicate with any other. This operation, which allows processors to move data to and from one another's memories, is similar to 'indirect addressing' on a conventional machine. Using this mechanism, the system is able to move  $\sim 2.5 \times 10^8$  32-bit words of data per second.



**Fig. 1** 'Digital Orrery' algorithm shown schematically for  $N = 5$ . Messages with positions and masses are passed around the ring, the data from each particle visiting all  $N - 1$  other particles.

The overall flow of information during these communication cycles may be divided loosely into fixed pattern and general pattern communications. Fixed pattern communications come up regularly in scientific computations; for example, filtering a two-dimensional image may require a fixed two-dimensional pattern of communication, or simulating the flow of a fluid by fixed-grid numerical methods may require a fixed three-dimensional pattern. Such regular motion of data between cells representing an  $n$ -dimensional grid is an operation supported directly by most fine-grained machines. These fixed pattern communications take place in approximately the same time as required for addition.

Another common fixed pattern of communications is that of a binary tree. An efficient method, for example, for computing the total kinetic energy of all of the stars in the system, is first to add the energies in each of 32,768 pairs of stars, and then again add each of 16,384 pairs of pairs, and so on. Each of these operations require a single step in a parallel machine, so 15 steps are required to add the total energy of all 65,536 ( $2^{15} + 1$ ) stars. The communication structure of such a computation is a 15-deep binary tree. Computations of this type are also supported directly by the Connection Machine's communication network. A related communications structure is the 'perfect shuffle' pattern of the fast Fourier transform. This is also supported by the hardware. In the fixed-pattern communication the pattern of data motion is determined by the algorithm, rather than by the data.

In many scientific calculations, the pattern of communications is data-dependent. For example, in efficient algorithms for inverting large sparse matrices, the pattern of communication will depend upon the pattern of non-zero entries in the matrix. Equivalently, in solving a loosely-coupled system of differential equations using numerical methods, the communications patterns will depend on which variables interact. Another example occurs in simulating the flow of a fluid with a non-uniform grid so that portions with more detailed structure are simulated to a higher degree of accuracy. Each of these examples requires motion of data between cells in an irregular pattern that depends on the particular structure of the problem. This type of data-dependent communication is supported on the Connection Machine through the use of indirect addressing. If one processor stores the address of another, then the first processor is able to access the memory of the other through the communications network. An arbitrary pattern of connections between the processors can be created by storing the appropriate pattern of addresses.

In some scientific applications the natu-

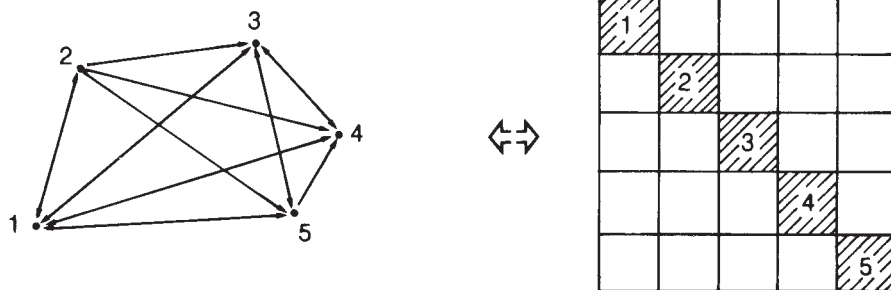


Fig. 2 'Simultaneous' force calculation algorithm for  $N = 5$ . All  $N(N-1)$  interactions are computed in parallel (open squares on checkerboard).



Fig. 3 'Hierarchical' force calculation algorithms. In *a*, particles (solid circles) receive messages directly from other particles and from centroids (open circles) representing distant groups of particles. In *b*, a variation in which long-range interactions computed directly between centroids (thick arrows) are shown, as well as communication between particles (thin arrows).

ral communications pattern changes during the course of the computation. For example, in calculating the flow of fluid it may be best to use an adaptive grid method where more processing is assigned to an area of space whenever turbulence occurs. The adaptive grid 'patches in' a finer mesh structure only where, and when, it is needed. The Connection Machine supports this dynamic storage allocation. Assume that some processors (Subset F), are kept free for this purpose. Other processors (Subset S) are identified as needing assistance at a given moment. Pointers to one or more free processors are set up in each processor in subset S, so that processors in S can use the communications router to distribute portions of the work to the newly allocated processors. This type of allocation of free processors is supported as a primitive operation.

### The $N$ -body problem

As an example of a scientific computing problem which may take advantage of a parallel computer, we consider one of the oldest problems in mathematical physics, the evolution of a system of  $N$  self-gravitating bodies. One idealization of this problem is expressed in the following equations of motion:

$$\frac{d^2 \mathbf{r}_i}{dt^2} = \sum_{j \neq i} \frac{G m_j (\mathbf{r}_j - \mathbf{r}_i)}{|\mathbf{r}_j - \mathbf{r}_i|^3} \quad i = 1, \dots, N \quad (1)$$

where  $m_i$  and  $\mathbf{r}_i$  are the mass and position respectively of body  $i$  and  $G$  is the gravitational constant. The formulation and solution of this problem for a system of  $N = 2$

bodies was one of the first major triumphs of newtonian mechanics. For  $N \geq 3$  no general solution is known. Over the past 300 years considerable progress has been made in developing analytic approximations for special cases, including the Solar System. For large- $N$  systems such as star clusters ( $N \approx 10^6$  stars) and galaxies ( $N \approx 10^{11}$ ), only a handful of equilibrium solutions are known, and many questions about the existence, stability, dynamical evolution, and interactions of such systems pose intractable mathematical problems.

With the introduction and steady improvement of digital computers, numerical simulations of so-called ' $N$ -body models' governed by equation (1) have become an increasingly important tool for studying the dynamics of self-gravitating large- $N$  systems. (For many purposes in the study of large- $N$  systems, an approximation of the exact  $r^{-2}$  force law in (1) may be acceptable. One standard approximation, which has the advantage of removing the singularity when  $r = 0$ , is to replace the denominator in (1) with  $(|\mathbf{r}_j - \mathbf{r}_i|^3 + \epsilon^2)^{3/2}$ , where  $\epsilon$  is a small 'softening' parameter with dimensions of length.) Such simulations tend to be computation-intensive, because evaluating of the summation in (1) requires  $O(N)$  operations to obtain the force on one particle. A naive  $N$ -body code therefore takes  $O(N^2)$  operations per timestep. Alternatively, the gravitational potential may be expanded in a suitably chosen set of basic functions. Algorithms of this kind require significantly fewer operations.



However, potential-expansion codes often lack generality and suffer from limitations on the range of length-scales which may be studied.

Conventional  $N$ -body algorithms that explicitly loop over all bodies  $j = 1, \dots, N$  to evaluate the force on body  $i$  may be adapted to use a Connection Machine with relatively trivial modifications. One processor is used for each body, so that the forces on body  $i$  due to each body  $j \neq i$  are computed simultaneously in processors  $1, \dots, i-1, i+1, \dots, N$ . The total force on body  $i$  is then obtained by adding up these forces, an operation which may be accomplished in time  $O(\log N)$  using a fixed binary-tree communication pattern.

## Parallel algorithms

More interesting are  $N$ -body algorithms designed for parallel machines. These algorithms can offer significantly higher levels of performance; they also reveal much more of the methodology of parallel computing. Here three examples are presented. These algorithms are described in terms of systems of abstract machines communicating with each other in various patterns, all of which may be efficiently implemented on a Connection Machine.

First, we consider the 'Digital Orrery' algorithm<sup>4</sup>, in which one processor is assigned to each particle, and all  $N$  particles are linked together in a ring, as in Fig. 1. Positions and masses of particles are passed around the ring. All processors execute the same basic step: forward a message to the next processor on the ring, and compute a two-particle interaction using the message received in turn. A complete force calculation requires  $N-1$  such steps, after which  $N(N-1)$  interactions have been evaluated. Velocities and positions may then be updated using any one of a number of possible integrators (for example, leapfrog, Runge-Kutta, predictor-corrector), and the whole process is then repeated. The main advantage of this approach is simplicity; the main disadvantage is the limited scope for improvement on the basic algorithm. The timestep must be short enough to resolve the shortest timescale of dynamical interest, which may be very small compared to the overall evolution time of the system. With a high-order integrator, the numerical error (usually measured by the change in the calculated binding energy, which should be conserved) may then be made quite small; however this leads to only a small increase in the timestep for a fixed level of permissible error.

A simple code of this kind with a time-centred leapfrog integrator has been tested on a Connection Machine. For  $N \approx 2,000$  particles, a timestep takes  $\sim 1$  min, with each processor computing  $\sim 30$  newtonian two-body interactions per second.

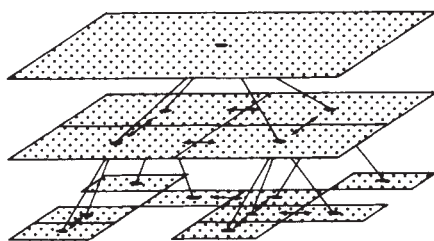


Fig. 4 The two-dimensional analogue of the 'Eulerian' tree structure used in the hierarchical algorithms, showing how the root cell (top) is recursively divided into smaller cells. Here,  $N = 9$ .

The timing provides the basis for most of the estimates made here. A typical calculation spanning a few dynamical times will require  $\sim 500$  timesteps, so the practical limit is  $N \approx 4,000-8,000$  if interesting calculations are to run in  $\sim 1$  day. Such a calculation would only use a fraction of the 65,536 processors on a full-sized Connection Machine. To use a 65,536-processor machine efficiently, we can run 8-16 such calculations at the same time, exploiting parallelism at the level of experiments.

A second algorithm is in the 'Simultaneous Force Calculation' algorithm. Instead of assigning one processor per particle,  $O(N^2)$  processors may be used to compute all two-particle interactions simultaneously. In the simplest version, sketched in Fig. 2, processors are laid out in an  $N \times N$  grid, with those on the diagonal representing particles. At the beginning of a force calculation, each particle processor sends its current position to all other processors in the same row and column. Each off-diagonal processor then computes a single two-body interaction. The resulting forces are summed up and used to advance particle coordinates with a standard numerical integrator.

A non-local communication pattern is required to implement this algorithm. Both the broadcast and the subsequent force summation would take  $O(N)$  steps if implemented naively by the one-dimensional communications pattern as used in the Orrery algorithm. These tasks can be accomplished in  $O(\log N)$  steps with fixed binary-tree communications patterns. The simultaneous force calculation algorithm can attain very high speeds when applied to relatively small- $N$  systems. For example, a system of  $N = 256$  particles would run at a rate of  $\sim 16$  timesteps per second on a Connection Machine; a typical calculation might require half a minute. Larger systems can be run by using each physical processor to simulate several 'virtual' processors, an option supported transparently by the Connection Machine. The limit with existing hardware is probably  $N \approx 1,000$ . A fast code of this kind can be very useful in exploring the parameter space of interest before performing large-scale calculations.

Third, there are 'Hierarchical Force

Calculation' algorithms, in which the total force on a particle may be approximated by summing over a subset of  $O(\log N)$  interactions instead of the full  $N-1$  (ref. 5.) Two possible versions of this approach are illustrated in Fig. 3. On the left, we show a scheme in which each particle receives messages from (1) particles which lie nearby, and (2) centroids that represent many particles grouped together using a centre-of-mass approximation. As messages come in they are used to calculate accelerations, which are added to a running sum. On the right, we show an approach in which long-range two-body interactions are computed directly between centroids, and local interactions take place between particles. The total acceleration on a particle then includes the partial terms collected by centroids that claim that particle as a member.

## Eulerian tree

The flow of messages required to implement these hierarchical algorithms may be organized on a tree structure, in which leaves represent particles and interior nodes represent the centroids. On a Connection Machine, this tree structure employs one processor for each node. Following Barnes and Hut<sup>5</sup>, an 'Eulerian' tree that divides space into a nested hierarchy of cubical cells (Fig. 4) is constructed, starting with the particles and grouping nodes together into larger cells. The tree is regenerated after each integration timestep, ensuring that it accurately reflects the particle positions. The regular structure of the tree permits the message flow patterns to be specified in purely geometrical terms, greatly simplifying the design of these algorithms. Messages move through the tree in two directions: laterally between nodes on the same level (through auxiliary cross-links), or vertically from interior nodes toward descendant cells and particles. Each node broadcasts its mass and centre-of-mass position laterally to a limited neighbourhood of nearby nodes. On receiving this information, a cell may forward it vertically to be consumed by descendant particles; this implements the version shown in Fig. 3a. Alternatively, messages received by a cell from other nodes on the same level may be consumed locally to obtain a partial acceleration, which is subsequently added to partial accelerations from other levels of the tree to obtain the total force on the particles; this implements the scheme shown in Fig. 3b.

The speed of these algorithms depends on the choice of neighbourhood, which may be varied to trade computational throughput for accuracy. With a preliminary implementation of the algorithm shown in Fig. 3a, a force calculation for  $N = 32,768$  particles takes  $\sim 5$  min; as expected, this time scales only as  $O(\log N)$  (ref. 7). Interesting calculations may be

run in a few hours to a day. The variation shown in Fig. 3b is likely to be an order of magnitude faster, although the additional approximation involved in this scheme must be carefully studied before a conclusive comparison can be made. With virtual processors, systems of up to  $N = 2^{18}$  particles could be simulated with existing hardware. For an application requiring a guaranteed level of accuracy, a parallel version of an adaptive multipole algorithm could be used<sup>6</sup>. The communications requirements would be similar.

## Applications

The scientific applications of these algorithms are extensive. Problems such as collisions between disk galaxies require a very large number of particles (to model the disk) and high spatial resolution (to

handle a wide range of scales). Very few self-consistent collisions between disk + halo galaxies have been attempted. Other possible applications include studies of dynamical stability and violent relaxation.

The self-gravitating body problem fits well on a fine-grained parallel computer because, like so many other computations in science, it is inherently parallel. Since the physical world operates in parallel, parallel algorithms are often closer to reality than their serial counterparts. It may be that parallel computers will be easier to program than the sequential machines. Parallel computers will almost certainly become more powerful and more widely accessible in the near future, so we will soon have the opportunity to learn.

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## REVIEW ARTICLE

# Water on Mars

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*Estimates of the amount of water outgassed from Mars, based on the composition of the atmosphere, range from 6 to 160 m, as compared with 3 km for the Earth. In contrast, large flood features, valley networks, and several indicators of ground ice suggest that at least 500 m of water have outgassed. The two sets of estimates may be reconciled if early in its history, Mars lost part of its atmosphere by impact erosion and hydrodynamic escape.*

THE amount of water outgassed from Mars has been a controversial issue ever since 1972 when the Mariner 9 spacecraft returned pictures of large channels incised into the planet's surface. The controversy stems in part from an apparent discrepancy between evidence from the surface which suggests that abundant water has outgassed and evidence from the composition of the atmosphere which suggests that little water has outgassed. A reconciliation appears underway in favour of the higher estimates. The reconciliation has been facilitated by a decade of analysis of Viking data, by recognition that the SNC meteorites probably come from Mars and by identification of several processes whereby a planet may lose part of its early atmosphere. The amount of water at the surface is of considerable geological, climatological and biological interest. Water plays a crucial role in many geological processes, including both deep-seated magmatic processes and surficial changes such as weathering and erosion. Because most of the water is believed to have outgassed early on, the near-surface inventory also provides clues about the accretion of the planet, the thermal and fractionation history of the interior, and the formation of the atmosphere. The amount of water also has climatological implications which in turn affect estimates of the probability that life could have started on the planet. There may even be practical implications, for water is a potential resource that future explorers could use for fuel and sustenance. This article summarizes the evidence on which the current inventory of water is estimated, suggests where the water may now be, and how its distribution may have changed with time.

Assessment of the amount of water present is severely impeded by the stability relations of water under current climatic conditions. With a mean atmospheric pressure (due to CO<sub>2</sub>) of 6 mbar

and mean diurnal temperatures that range from 150 K at the winter pole to 220 K at low latitudes, liquid water is unstable everywhere on the surface, ice is stable only at the poles<sup>1,2</sup>, and a thick permafrost zone ranges in thickness from several hundred metres at the equator to kilometres under the poles<sup>3-5</sup>. The only place on the surface where water ice has been unambiguously identified is at the north pole where evaporation of the seasonal CO<sub>2</sub> cap in summer exposes the water ice<sup>6,7</sup>. Ground ice is stable at depths greater than several centimetres at latitudes above about 40° (refs 1, 2). At lower latitudes, however, ground ice is unstable at all depths, and will sublime into the atmosphere at rates dependent on the porosity of the overlying materials. Although the atmospheric pressure may change cyclically because of obliquity variations<sup>8-10</sup>, such changes are unlikely to be large enough to alter significantly the stability relations just described<sup>11</sup>. As a consequence, the near-surface materials at low latitudes should, over geological time, have lost any unbound water down to depths of tens to hundreds of metres<sup>12,13</sup>. Such losses could, however, be partly replenished by outgassing of water from greater depths or by subsurface migration from higher latitudes<sup>14</sup>.

The column abundance in the atmosphere ranges from less than 5 precipitable micrometres (pr.  $\mu\text{m}$ ) in the winter hemisphere to over 100 pr.  $\mu\text{m}$  over the northern summer pole<sup>2</sup>. The average content is about 10 pr.  $\mu\text{m}$ . Although these amounts are very small, the lower 1 km of the atmosphere is close to saturation for night temperatures<sup>15</sup>. The seasonal variations in water content of the atmosphere are due to exchange with the regolith, trapping and release of water in the seasonal CO<sub>2</sub> cap, and loss from the permanent northern water-ice cap<sup>15</sup>.

Climatic factors thus prevent liquid water at the surface, allow





**Fig. 1** The 18-km diameter crater Yuty (22° N, 34° W). The ejecta are arrayed in discrete lobes that appear to have flowed outward away from the crater. The lobes are in sharp contrast to the disorganized blocky debris, typical of lunar craters. The fluidity of the debris has been ascribed to entrained water. Such patterns are almost universal around Martian craters larger than 3–5 km possibly because these craters penetrate the thick permafrost.

ice at the surface only at the poles, and result in only minute amounts of water in the atmosphere. If the planet has outgassed large amounts of water, most must be hidden from view either as ground ice or ground water. As only the surface and atmosphere are accessible to direct measurement, the water inventory must be assessed indirectly. This has been done in three basic ways, from geological evidence, from the composition of the atmosphere, and from models of planet formation.

### Early views

Our perception of the amount of water at the Mars surface has ranged widely over the last three decades. During the 1950s the atmospheric pressure at the surface was believed to be 100–250 mbar, and due largely to nitrogen. Abundant water was assumed present, and it was thought possible that seasonal changes in albedo and colour could be due to biological activity. This view of Mars was shattered in the early 1960s by measurements of surface temperatures that were inconsistent with any significant greenhouse warming<sup>16</sup>, by spectroscopic measurements which showed that the surface pressure was close to 4 mbar (ref. 17) and that only about 15  $\mu\text{m}$  of water was present in the atmosphere<sup>18</sup>, and by pictures from the Mariner 4 spacecraft that showed a lifeless cratered surface, much like that of the Moon<sup>19</sup>. This new view of Mars as an inert body, devoid of any evidence that liquid water had been on its surface, appeared confirmed by Mariners 6 and 7. But our perception changed again when, in 1972, the Mariner 9 spacecraft sent back numerous pictures of large channels, which appeared to be clear evidence that large amounts of liquid water had flowed over the surface<sup>20,21</sup>. By this time the stability relations of water, outlined above had been well established<sup>1</sup>, so the channels were viewed as evidence for major climate changes in the past<sup>22</sup>. Although the geological evidence suggested that Mars had outgassed significant amounts of water, no quantitative estimates

were made of the amount at that time. The supposition that Mars could have outgassed large amounts of water was consistent with most current models for the formation of the planets which predicted that at least the initial inventory of volatiles should increase with increasing heliocentric distance. This appeared true whether accretion was homogeneous<sup>23</sup> or inhomogeneous<sup>24</sup>, and whether the density differences between the terrestrial planets are due to fractionation of silicate and metal particles<sup>25</sup> or the oxidation state of the accreted materials<sup>26</sup>. Just before the Viking mission, it was argued from geochemical evidence that Mars had outgassed as much water as the Earth<sup>27,29</sup>, but these arguments were based on a substantial overestimate of the abundance of argon in the atmosphere.

### Estimates from atmospheric composition

Measurement of the composition of the Martian atmosphere<sup>30</sup> by the Viking landers provided a means of rigorously estimating the amount of water outgassed. Estimates were made in two ways, from the isotope composition of nitrogen and oxygen in the atmosphere and from its noble gas content. From the lack of fractionation of oxygen isotopes McElroy *et al.*<sup>31</sup> showed that the atmosphere must exchange oxygen with some surface reservoir. Assuming the reservoir is near-surface water, they estimated that it must contain at least 13 m of water averaged over the planet; otherwise they would have observed <sup>18</sup>O enrichment in the atmosphere. They also assessed the outgassed water from the enrichment of the atmosphere in <sup>15</sup>N. The enrichment occurs because <sup>14</sup>N is preferentially lost from the upper atmosphere as a result of photodissociation. Reconstruction of the initial inventory of nitrogen is uncertain. It depends on the escape efficiency of nitrogen, isotope fractionation in the upper atmosphere, and the rate of fixation of nitrogen in the surface as nitrates and nitrites, which are all poorly known, but the best estimate of McElroy and co-workers is that the initial surface pressure of N<sub>2</sub> was 20 mbar as compared with the present 0.15 mbar. This implies 120 m of water were outgassed if the H<sub>2</sub>O/N ratio was initially the same as on Earth.

Anders, Owen and Morgan<sup>32,33</sup> used the noble gas content of the atmosphere to estimate the outgassed water. The bulk potassium content of the planet was estimated to be between 62–100 p.p.m. from measurements of the radioactivity of surface rocks<sup>34</sup>, and from constraints on internal heat production implied by thermal models of the interior<sup>35</sup>. From the bulk potassium content, the amount of <sup>40</sup>Ar produced over the history of the planet was calculated, and this was compared with that in the atmosphere to derive an estimate of the fraction of <sup>40</sup>Ar released to the atmosphere. This release-factor was estimated to range from 0.067 to 0.099. Since <sup>36</sup>Ar was incorporated into the planet during accretion, its release-factor must be at least as large as that of <sup>40</sup>Ar. If we assume that all the <sup>36</sup>Ar released is still present in the atmosphere, then upper and lower limits can be placed on the global inventory of <sup>36</sup>Ar from the present content of the atmosphere. Even the upper limit on the global inventory, which assumes equal outgassing of <sup>36</sup>Ar and <sup>40</sup>Ar, was estimated as 17 times less than that of the Earth. Anders, Owen and Morgan concluded, therefore, that Mars is considerably less rich in volatiles than the Earth. The global water inventory was estimated by assuming that all volatiles are present in the same proportions as in C3V chondrites. Finally, the amount released to the surface, estimated at 9.4 m averaged over the whole planet, was determined by narrowing further the release-factor by comparisons with <sup>129</sup>Xe and by arguing that the release-factor of nitrogen should be no greater on Mars than on the Earth. Rasool and LeSargeant<sup>36</sup>, by similar reasoning, but using volatile ratios in ordinary rather than C3V chondrites, estimated that 6 m had been released. For comparison, the Earth is estimated to have outgassed 3 km of water<sup>24</sup>. The abundances derived from the noble gases are thus very low, contradicting previous estimates of Earth-like abundances, and implying that both the surface and the planet as a whole are very volatile poor.



**Fig. 2** A section of the outflow channel Mangala Vallis, believed to have been formed by large floods. Branching channels are incised into the surface to depths of several hundred metres. Individual branches are tens of metres across and the entire scene is 100 km across. The large volume of material eroded to form the channels suggests large volumes of water have flowed through them.

Subsequent discovery that the  $^{36}\text{Ar}/^{12}\text{C}$  ratio is 20–200 times larger in the Venus atmosphere than in the volatiles outgassed from the Earth led Pollack and Black<sup>37</sup> to question that the  $^{36}\text{Ar}/^{12}\text{C}$  ratio can be assumed constant in all the terrestrial planets, a basic assumption in the estimation of water on Mars from noble gases in the atmosphere. To explain the Venus results Pollack and Black suggested that different mechanisms controlled incorporation of the noble and non-noble gases into the planets during accretion. Noble gases were mostly adsorbed on grains, whereas other volatile elements were mostly chemically bound within the grains. They suggested that while the planets were accreting, the solar nebula was essentially isothermal with the nebular pressure decreasing outwards. As adsorption depended on the local pressures, Venus accreted from grains with more adsorbed noble gases than did the Earth, which in turn was formed of material with a higher noble gas content than Mars. In contrast, they suggested that the incorporation of volatiles such as  $\text{CO}_2$ ,  $\text{N}_2$  and  $\text{H}_2\text{O}$  was essentially independent of position. Assuming equal outgassing efficiencies for  $^{14}\text{N}$  and  $^{36}\text{Ar}$  on Mars, they deduced that the original  $^{14}\text{N}/^{36}\text{Ar}$  on Mars was 40 times that on the Earth. Then, from the amount of Ar and N outgassed, they deduced that Mars had outgassed less efficiently than the Earth, and that 80–160 m of water were present at the surface. Thus estimates from the composition of the atmosphere suggest that Mars has outgassed from 6 to 160 m of water, proportionately 20–500 times less than the Earth.

## Geological evidence

The surface of Mars has a variety of features that suggest it is water or ice rich. These include pitted ground that resembles terrestrial thermokarst, polygonally fractured ground, debris flows, craters with central pits and craters with lobate ejecta patterns<sup>4,5,38,39</sup> (Fig. 1). But channels provide the most obvious evidence for large amounts of water close to the surface. Immediately upon their discovery in 1972, the channels were recognized as probably having been formed by running water<sup>20,21,40</sup>. This interpretation is now almost universally accepted, although ice action and mass wasting may have been contributing factors in many cases. Alternative proposals that do not involve ice or water, such as faulting, and erosion by wind, lava or liquid

hydrocarbons are all implausible for a wide variety of reasons<sup>41,41</sup>. Three main types of channels have been recognized: outflow channels, valley networks and fretted channels<sup>42</sup>. Because they lack tributaries, start full size, and have numerous attributes in common with terrestrial flood features, most of the outflow channels are believed to have been formed by large floods<sup>40,41,43,44</sup>. Some of the floods must have been enormous, having scoured swaths of terrain tens to hundreds of kilometres wide (Fig. 2). From the channel dimensions, Baker<sup>41</sup> estimates that discharges ranged as high as  $3 \times 10^8 \text{ m}^3 \text{ s}^{-1}$ . For comparison, the average discharge of the Mississippi is  $3 \times 10^4 \text{ m}^3 \text{ s}^{-1}$ . The cause of the floods is uncertain, and all probably did not form in the same way. Among the possibilities are volcanic interaction with ground ice<sup>45,46</sup>, geothermal melting of ground ice<sup>20</sup>, geothermal decomposition of hydrated minerals<sup>47</sup>, and eruption of water under high pressures from confined aquifers<sup>48</sup>. All the plausible mechanisms imply abundant subsurface water either as ice or liquid. Because of the large discharge rates, these channels could form under present climatic conditions<sup>49</sup>.

The total volume of water that flowed through the channels is difficult to determine, but rough estimates can be made from the amount of erosion that has been achieved. The largest outflow channels are round the Chryse basin, where about  $5 \times 10^6 \text{ km}^3$  have been eroded to form channels, chaotic terrain and some of the smaller canyons<sup>49</sup>. The main east-north-east to west-south-west canyon was ignored in this estimate because it appears to be formed in large part by faulting<sup>50</sup>. The estimates of the eroded volumes could be in error by as much as a factor of two because of uncertainties in the channel depths. The minimum amount of water required to perform this erosion is estimated to be  $7.5 \times 10^6 \text{ km}^3$  on the assumption that all the water carried its maximum sediment load, or 40% by volume<sup>51</sup>. This is a conservative assumption, implying that the terrain offered little resistance to erosion, and that most of the eroded sediment was transported as wash load at very high concentrations. The amount of water is equivalent to 50 m spread over the whole planet.

To determine what this volume implies about the total surface inventory, we must make some judgement about whether outflow channels started at specific locations because that was where water was especially abundant, or whether they started there because conditions necessary for rapid release were achieved. The distribution of valley networks suggests the latter is more likely. The valley networks resemble terrestrial river valleys in that they increase in size downstream and form integrated drainage networks (Fig. 3). They appear to have formed by slow erosion rather than by floods<sup>41,52,53</sup>, and largely as a result of groundwater sapping rather than surface runoff<sup>54</sup>. About two-thirds of Mars is covered by an ancient cratered upland that is almost certainly underlain by an impact-generated megaregolith. By analogy with the Moon, this probably has a high porosity, and consequently a large potential capacity for ground water<sup>55</sup>. Most of the valleys are incised into the ancient cratered upland and appear themselves to be very ancient<sup>52,53,56,57</sup>, although there are rare exceptions<sup>58</sup>. Because of the difficulty of maintaining small streams under present climatic conditions, the valley networks have been taken as evidence that climatic conditions were significantly different early in the planet's history<sup>59,60</sup>. The distribution of the valley networks is almost uniform throughout the cratered terrain<sup>52,53</sup>. If formed mostly by groundwater sapping, they indicate that groundwater (or ground ice) was present everywhere in the cratered uplands very early in the planet's history.

I have suggested that floods were common in the region south and west of the Chryse basin because the large surface relief to the west of Chryse caused migration of ground water below the permafrost<sup>48</sup>. The consequent build-up of hydrostatic pressures caused catastrophic breakout from below the permafrost to form the floods. From the local relief and from the chaotic terrain, which provides evidence of withdrawal, we can outline the area



that was drained to form the floods, and this constitutes about one tenth of the planet's surface<sup>49</sup>. Assuming, on the basis of the distribution of channel networks, that ground water was initially distributed evenly, we can extrapolate the volume of ground water released to form the Chryse channels to the entire planet, thereby obtaining a planet-wide inventory of 500 m. This figure is conservative because of the maximum sediment load assumption and because it is unlikely that all the ground water in the Chryse region came to the surface to form floods. The estimate would, however, be invalid if there were some re-cycling mechanism whereby the same water participated in the flooding several times<sup>14</sup>.

Almost all of the outflow channels terminate in low-lying plains at high northern latitudes. These plains have numerous features that have been attributed to the presence of ground ice<sup>4,38,39</sup>. Included are polygonally fractured ground, irregular depressions that resemble terrestrial alases, table mountains similar to those in Iceland, and arrays of sub-parallel ridges and pits. The flood-waters probably pooled and froze in these areas. Most of the water may still be present as ice, protected by a debris cover that accumulated as the sediment-laden ice near the surface sublimed.

The above argument assumes that the water that eroded the Chryse outflow channels erupted from below the permafrost, estimated to be nominally 1 km thick. The amount of water existing as ice at shallower depths can be estimated from features in the fretted terrain and from 'terrain softening'. Fretted terrain is the term applied to areas where relatively flat lowlands are separated from the cratered uplands by a complex pattern of escarpments<sup>61-63</sup>. The main occurrence is where the boundary between the cratered uplands and the younger plains crosses the 30-50° N latitude band. Debris flows extend up to 30 km from the base of escarpments throughout the terrain (Fig. 4). In this latitude band, talus apparently moves away from slopes as a debris flow, instead of accumulating as a stable deposit at the base of slopes, as it does elsewhere. The simplest explanation of the enhanced mobility is that at the higher latitudes the talus contains ice<sup>62,63</sup>, and that the ice is derived from the cratered uplands that are being eroded. Presence of abundant ground ice at high latitudes is also indicated by a general muting of the topography, which is also observed mainly in the 30-50° latitude bands<sup>64</sup> (Fig. 5). Here, crater rim crests are rounded, fine texture on ejecta blankets is lost, and the terrain between craters has a smooth rolling character rather than the crisply defined ridges

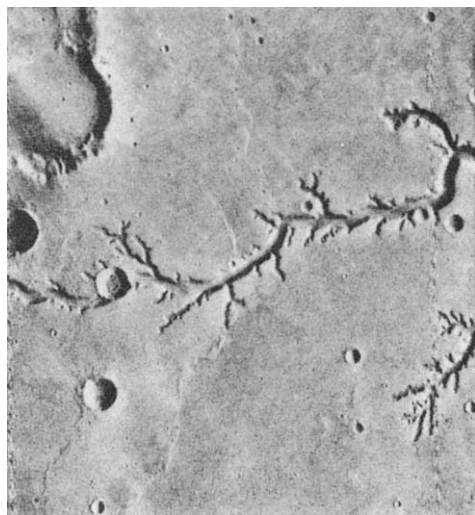


**Fig. 4** Debris flows surrounding remnants of the cratered uplands at 45° N, 322° W. The mobility of the flows is thought due to entrainment of ice shed from the cliffs at their source<sup>64</sup>. The picture is 90 km across.

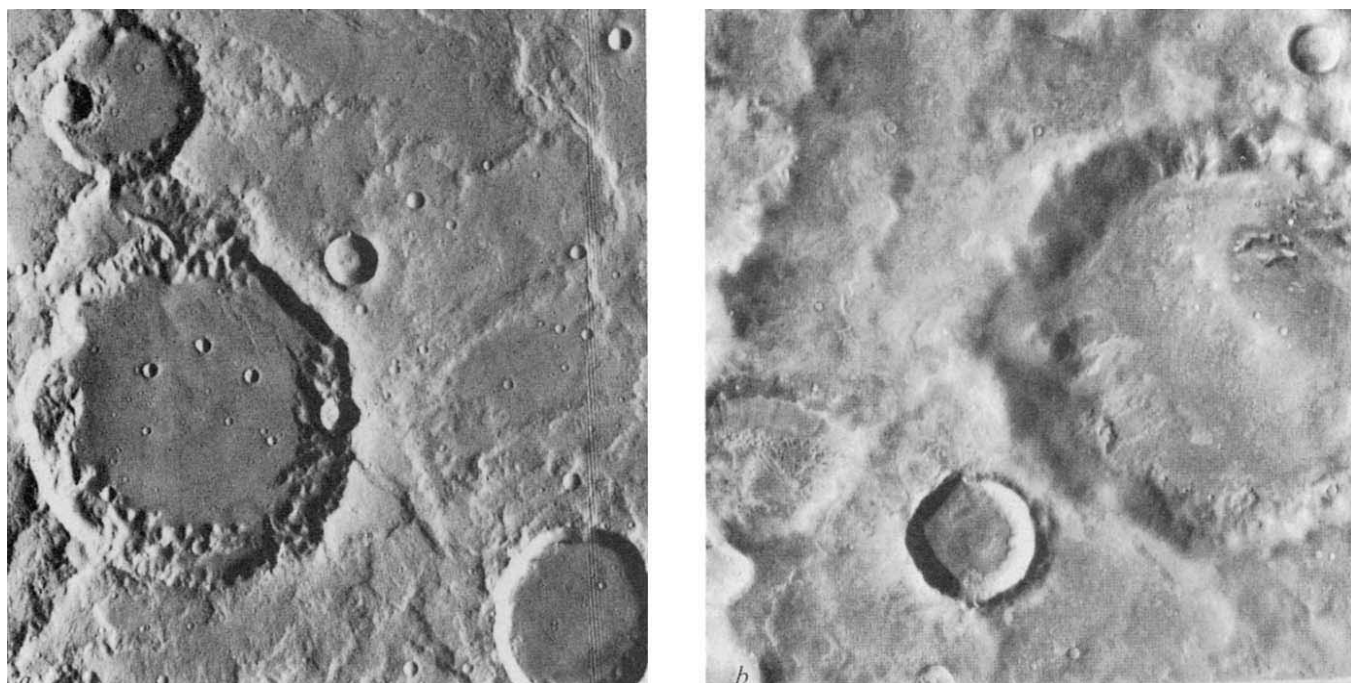
and hills found at lower latitudes. Again, the most probable explanation of the softening is that it is caused by creep of the near-surface materials as a result of the presence of ground ice. Absence of debris flows and softening at low latitudes result from instability of ground ice at these latitudes and its consequent loss by sublimation. Absence of debris flows and softening at very high latitudes may result from the higher effective viscosity of ice at the low ground temperatures there<sup>63,64</sup>. The amount of ice required to mobilize the talus and to cause the softening is unclear, but analogy with terrestrial rock glaciers, and arguments based on the requirement that flow of ice rather than friction between the rock component controls the viscosity, suggests at least 10% ice is required<sup>49,63</sup>. If no ice is present at latitudes less than 30° and 10% is present at higher latitudes, then the upper 1 km contains 50 m of unbound water averaged over the whole planet. The geological evidence suggests, therefore, that at least 550 m of water have outgassed, substantially more than the estimates from the composition of the atmosphere, particularly those based on the noble gases.

### SNC meteorites

The SNC meteorites provide yet another way of estimating the amount of water at the Martian surface. The basaltic composition of these meteorites, their young ages, and the composition of included gases indicate that they almost certainly come from Mars<sup>65-67</sup>. Assuming that they did, Dreibus and Wänke<sup>68</sup> show that most of the moderately volatile elements (Na, K, Rb, Zn, Ga, Cl, Br, I) are enriched in the Martian mantle with respect to the Earth's by factors that range from 1.3 to 4. Unfortunately, the SNC meteorites give little direct information on highly volatile compounds such as CO<sub>2</sub> and H<sub>2</sub>O. To estimate these volatiles, Dreibus and Wänke invoked an accretionary model previously used to estimate the bulk composition of the Earth<sup>69</sup>. Following Ringwood<sup>70</sup>, they suggest that the Earth and Mars accreted from two components, a highly reduced, volatile-poor component A, similar to enstatite chondrites, and an oxidized, volatile-rich component B, similar to CI carbonaceous chondrites. The composition of the Earth's mantle is best matched by an A/B mixture of 85/15, whereas the moderately volatile elements in the Mars mantle imply it was formed from 65/35



**Fig. 3** The upper reaches of Nirgal Vallis, believed to have been formed by slow erosion of running water. The open nature of the tributary system, the lack of dissection between major branches, and their alcove-like terminations suggest origin by groundwater sapping. The scene is 80 km across.



**Fig. 5** Latitudinal contrasts in terrain morphology. *a*, Typical cratered upland at low latitudes (12° S). Rim crests of large craters are sharply defined, the small crater population is well preserved, and ridges between craters are clearly discernible. *b*, Typical cratered upland in the 30–55° latitude bands (48° S), showing terrain softening. Rim crests of large craters are rounded, the small crater population is poorly preserved and ridges between craters are muted. Both pictures are about 60 km across. The contrast between the two scenes has been ascribed to flow of the surface materials because of the presence of ground ice at the higher latitudes<sup>63</sup>.

mixture. The bulk composition implied by such a mixture matches well the composition inferred from the mean density and moment of inertia<sup>71</sup>. Dreibus and Wänke conclude therefore that Mars was initially a very volatile-rich planet, having incorporated over twice the fraction of the volatile-rich component as the Earth. They suggest, however, that Mars retained very little of its initial, very large water inventory, because the planet accreted homogeneously, and the water in component *B* reacted with iron in component *A* to produce hydrogen, which was lost. In contrast, the Earth retained a large fraction of its water because it accreted inhomogeneously as is indicated by incompatibilities between the core and the upper mantle<sup>70</sup>. Part of component *B* apparently was added too late to the Earth to react with component *A*. Assuming no water on the surface of Mars at the end of accretion, Dreibus and Wänke estimate, from the solubility of water in basaltic magmas, that the global water inventory is 51 m. The fraction that has outgassed and is now at the surface is unknown but their favoured value is 10–20 m.

### Current views and conclusions

We have just seen that estimates of the amount of water outgassed from Mars range over two orders of magnitude. The amount has major climatic implications, for if the lower figures are correct, then the planet has probably outgassed only about 0.1 bar of CO<sub>2</sub> and can never have experienced warm, wet, climatic conditions. If the upper figures are correct, then over 10 bars of CO<sub>2</sub> are likely to have outgassed, the planet may well have experienced warm, wet, conditions, and several biological implications may follow<sup>72</sup>. The geological evidence suggests that the lower estimates, those less than 100 m are extremely unlikely. The cumulative evidence of water erosion and ice-abetted processes is overwhelming<sup>4,5,39,41,42</sup>, even if computation of the amount from the geological evidence is suspect. How then can we reconcile the low estimates with the geological evidence? The lowest estimates are based on the SNC meteorites and noble gases in the atmosphere. The Dreibus and Wänke arguments for Mars being initially volatile rich are convincing, but the

supposition that Mars has lost all of its enormous initial inventory of water rests on the very questionable assumption of a complete planet-wide equilibration of its accreted components at the end of accretion. We know this is not true for the Earth<sup>70</sup>, and it seems unreasonable to insist that it be true for Mars. If one per cent of component *B* were accreted sufficiently late that it could not react with component *A*, then geological requirements for hundreds of metres of water could be satisfied.

Arguments for low water abundances based on the noble gases in the atmosphere rest on two questionable assumptions, that the materials that formed the terrestrial planets had a uniform ratio of noble to non-noble gases and that all the noble gases outgassed from the planet are still present in the atmosphere. We have seen from the Venus data that the first assumption is probably invalid. The second assumption is also suspect. Mars could have lost significant amounts of its early atmosphere by blow-off during large impacts<sup>73</sup>. The efficacy of such a process would depend on planet size, being more efficient on a planet like Mars, which has a relatively small gravitational field, than on the Earth. Loss would also preferentially favour the less reactive volatiles such as nitrogen and the noble gases, which tend to remain in the atmosphere, rather than volatiles such as CO<sub>2</sub> and H<sub>2</sub>O, which tend to be combined with surface materials. Part of the early atmosphere could also be lost by hydrodynamic escape<sup>73,74</sup> during the early massive hydrogen loss referred to by Dreibus and Wänke<sup>68</sup>. Thus the very low estimates of the water inventory appear doubtful on grounds other than their clear incompatibility with the geological evidence. The somewhat larger estimates of the water inventory from the nitrogen isotopes and from the accretion model of Pollack and Black are also suspect if impact erosion of the atmosphere has been significant. Blow-off would favour nitrogen over water, so that a common step in both arguments, use of the terrestrial N<sub>2</sub>/H<sub>2</sub>O ratio to compute the water inventory from the reconstructed nitrogen inventory, will lead to an underestimate of the amount of water present.

In summary, data from the SNC meteorites suggest that Mars



had an initial global inventory of volatiles that was proportionately larger than the Earth's. A major uncertainty is the fraction of these volatiles that has been outgassed and retained at the surface. Geological evidence suggests that at least 0.5 km of water have outgassed. On the reasonable assumption that CO<sub>2</sub>, H<sub>2</sub>O and N<sub>2</sub> were outgassed in terrestrial proportions, then 10–20 bars of CO<sub>2</sub> and 0.1 to 0.3 bars of N<sub>2</sub> also outgassed. Such a model can be reconciled with the geochemical data if the noble gases, and to a lesser extent nitrogen, were preferentially lost from the early atmosphere with respect to water. A plausible mechanism is impact erosion of the atmosphere. A large fraction of the water is present now as ground water and ground ice, well below the surface. Unbound water is present close to the surface only at high latitudes. Since the geological record emer-

ged around  $3.8 \times 10^9$  yr ago, the water has undergone some redistribution. That lost from the near surface materials at low latitudes by diffusion and seepage is probably now largely in the polar layered terrains. That involved in the formation of the large flood feature may still be at shallow depths in low-lying, high-latitude, northern plains. Indeterminate amounts of water may also be combined in weathering products, particularly clays. Because of the large amounts of CO<sub>2</sub> that were outgassed, early Mars may have possessed a thick atmosphere and experienced relatively warm climatic conditions. However, the fixation of CO<sub>2</sub> as carbonates caused the atmosphere to thin and surface temperatures to fall very early in the planet's history. Present climatic conditions are probably typical of what the planet has experienced for most of its history.

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## ARTICLES

# Multi-channel seismic imaging of a crustal magma chamber along the East Pacific Rise

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*A reflection observed on multi-channel seismic profiles along and across the East Pacific Rise between 8°50' N and 13°30' N is interpreted to arise from the top of a crustal magma chamber located 1.2–2.4 km below the sea floor. The magma chamber is quite narrow (<4–6 km wide), but can be traced as a nearly continuous feature for tens of kilometres along the rise axis.*

THE presence of a shallow crustal magma chamber at active spreading centres is an important element of current geological models for the formation of ocean crust<sup>1–3</sup>. However, the shape, longevity and along-strike variability of ridge-crest magma chambers remain the subject of considerably controversy<sup>4,5</sup>. The

feasibility of using modern multi-channel seismic techniques to image magma chambers at ridge crests was demonstrated a decade ago when three 24-channel seismic reflection profiles were obtained across the East Pacific Rise (EPR) near 9° N<sup>6,7</sup>. In May–June, 1985, we returned to this area and carried out a

much more extensive two-ship multi-channel seismic survey of the EPR between  $8^{\circ}50'$  N and  $13^{\circ}30'$  N. This experiment was designed to provide new information on the shape and dimensions of the axial magma chamber, and to investigate how the magma chamber varies along the rise axis over distances of tens to hundreds of kilometres.

The geological and tectonic setting of the EPR in this area has been well-established from Sea Beam and Sea MARC I surveys<sup>8-10</sup> extensive dredging along the rise axis<sup>11,12</sup>, and very high-resolution investigations using bottom photography and manned submersibles<sup>13</sup>. This portion of the EPR is spreading at relatively fast rates of  $5-6 \text{ cm yr}^{-1}$  (half-rate)<sup>14</sup> and contains one major transform fault (the Clipperton at  $10^{\circ}15'$  N), two large overlapping spreading centres (OSCs) at  $9^{\circ}03'$  N and  $11^{\circ}45'$  N and several smaller non-transform offsets including OSCs and other small deviations in axial linearity (Devals)<sup>9-11</sup>. Our seismic survey was primarily concentrated in two areas—between the  $9^{\circ}03'$  N OSC and the Clipperton transform, and in a second area just north of the  $12^{\circ}54'$  N OSC (Fig. 1). Seismic refraction data are available in both areas indicating the existence of a crustal low velocity zone (LVZ), interpreted as a magma chamber<sup>15-18</sup>, at the rise axis. However, our survey also encompasses part of the ROSE refraction area ( $\sim 11^{\circ}30'$  N to  $12^{\circ}30'$  N) where evidence for a large crustal magma chamber is generally lacking<sup>19,20</sup>.

The experiment involved two ships, the *Robert D. Conrad* of Lamont-Doherty Geological Observatory and the *Thomas Washington* of Scripps Institution of Oceanography. More than 3,500 km of conventional, 48-channel common-depth-point (CDP) reflection data were obtained including 30 new profiles across the EPR and a series of CDP reflection lines along the crest of the EPR between  $8^{\circ}50'$  N and  $13^{\circ}30'$  N (Fig. 1). The seismic velocity structure of the crust and upper mantle in the axial region was determined using the two-ship expanding spread profiling (ESP) technique<sup>21</sup>. The ESPs were oriented parallel to the rise axis and shot using airguns and, in some cases, explosives. In the  $9^{\circ}$  N area, ESPs were shot on the rise crest, and at two and five km away from the ridge crest on either side. In the  $13^{\circ}$  N area, five ESPs were located within  $\pm 3.6$  km of the rise axis.

Single-ship stacked time sections form the basis of our preliminary observations. About two-thirds of the cross-axis lines have been migrated after stack using the wave equation technique in the F-K domain<sup>22</sup>. Analysis of the ESPs has mainly involved P-wave velocity travel-time inversions on the airgun portions of the ESPs. An initial velocity-depth function was constructed using the tau-sum technique after transformation of the data into tau-p<sup>23</sup>. The velocity-depth function was then iteratively refined by forward travel-time and WKBJ synthetic seismogram modelling.

## Results

Two representative CDP reflection profiles across and along the EPR are shown in Figs 2 and 3. A large amplitude reflection is present about 0.6 s below the sea floor on both profiles. On the cross-ridge profile, this high-amplitude event is centred beneath the rise axis and is less than 3 km in width, although a weak continuation of this reflector appears to extend beneath the ridge flanks, especially west of the rise axis. An event is present at about 6 s two-way travel time that may be a Moho reflection based on its position in the section. On the reflection profile shot along the rise axis, a large-amplitude, relatively flat-lying reflector is present at about 4 s two-way travel time that corresponds to the event seen on the cross-ridge profiles. This event can be traced for distances of tens of kilometres along the crest of the EPR, and in a few locations, the amplitude of this reflector is strong enough that it can be observed on single channel monitor records. Some weaker, low frequency events are present below this strong reflection that may also be real, but no obvious Moho reflection is observed.

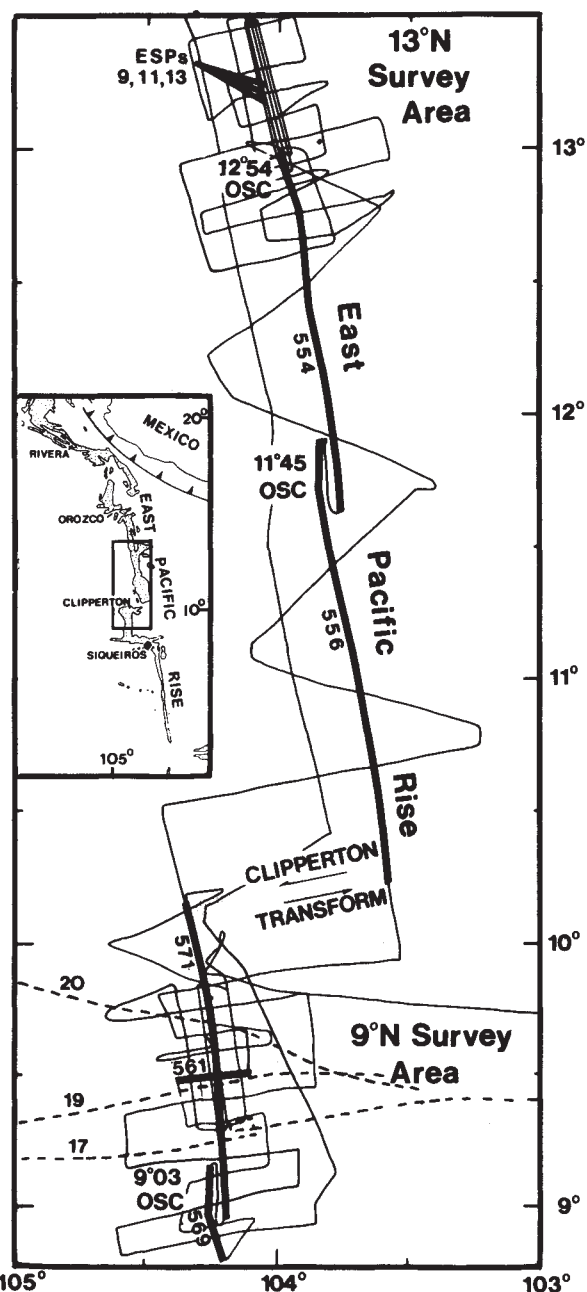
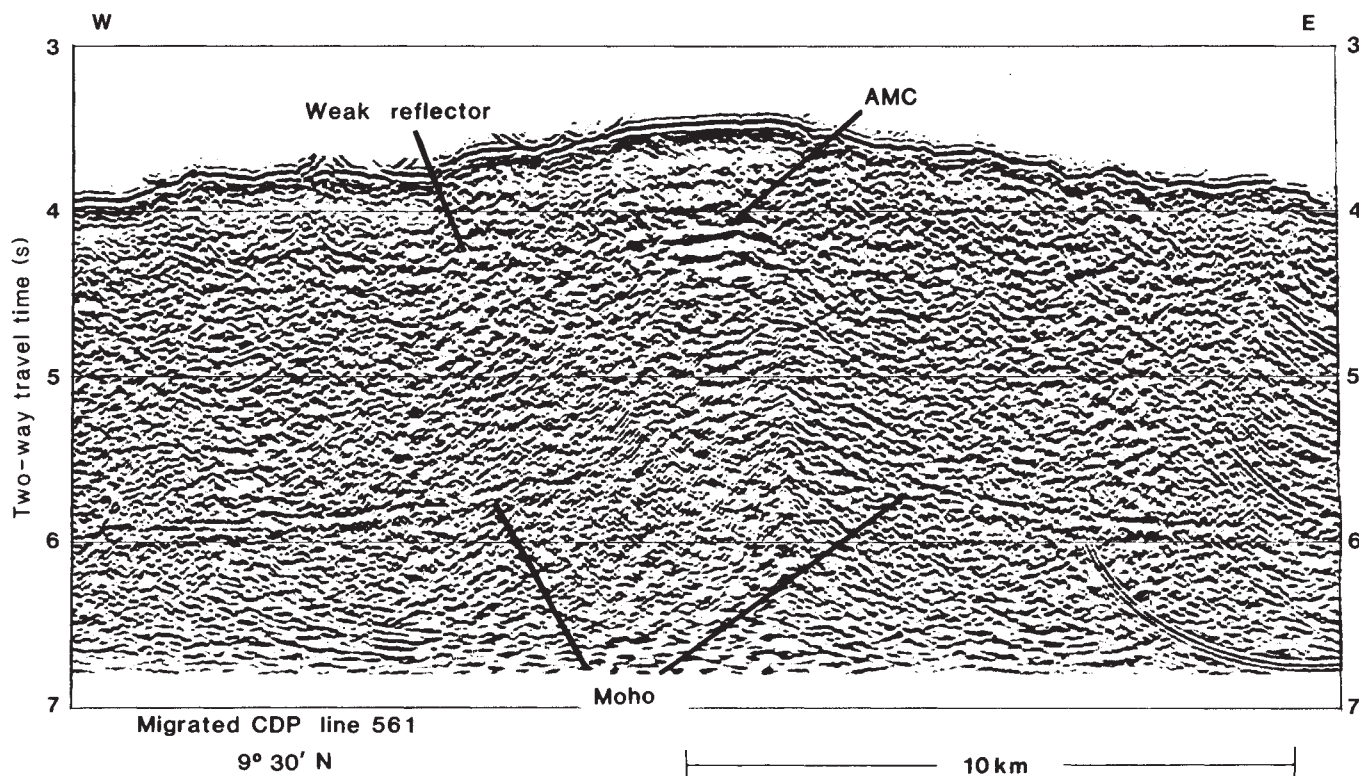


Fig. 1 Map showing the East Pacific Rise (EPR) and the track coverage obtained during our 1985 multi-channel seismic experiment. Labelled profiles (thick solid lines) are shown in Figs 2 and 3 or interpreted in Fig. 5. Dashed lines show the location of the reflection profiles described by Herron *et al.*<sup>6,7</sup>.

**Evidence for a crustal magma chamber.** Several lines of evidence indicate that the shallow event seen in these records at the rise axis is a reflection from the top of a crustal LVZ. The reflection is seen on single channel monitor records and in common mid-point gathers and thus is not an artefact of the stacking process. The stacking velocity of this event ( $2.3-2.4 \text{ km s}^{-1}$ ) is much higher than the value of  $1.5 \text{ km s}^{-1}$  expected for a water-path sidescatter event or a high-order water bottom multiple. This stacking velocity yields an interval velocity of  $4.9 \text{ km s}^{-1}$  for the crust above this reflector which is a reasonable average value for the upper 1 km of the oceanic crust near the EPR<sup>25</sup>. At several locations along the ridge, the polarity of this event observed on the near-offset trace in CDP gathers is clearly reversed relative to the sea floor return, as would be expected for a reflection from the top of a LVZ. However, the waveform





**Fig. 2** Migrated time section for CDP Line 561 which crosses the EPR near 9°30' N. These data were shot using a 29.3 l (1785 in<sup>2</sup>) airgun array and recorded on a 48-channel, 2.4-km streamer with an active group length of 50 m. The data were bandpass filtered 5–40 Hz, corrected for spherical divergence, predictively deconvolved (filter length 250 ms; prediction distance 40 ms), stacked and migrated using the F-K wave equation technique<sup>22</sup>. The data are displayed with a time variable gain and a 3-trace additive mix. The relatively flat, high-amplitude event beneath the rise axis is believed to be a reflection from the top of an axial magma chamber (AMC). A weak event at the same depth as the AMC reflector can be traced out beneath the ridge flanks and could represent the top of a frozen chamber. Moho reflections can be traced within 2–3 km of the rise axis.

is quite variable, even from CDP to CDP, and in other locations a phase-reversal is not apparent. The strongest evidence that this event is associated with an axial LVZ comes from the ESPs shot along the rise axis. They display a pronounced shadow zone for crustal arrivals beyond 11 km range that is very diagnostic of a LVZ<sup>18</sup> (Fig. 4). Although ESPs were obtained at only two locations along the ridge (9°35' N and 13°10' N), sonobuoys collected elsewhere along the rise axis also frequently display this same strong attenuation of refracted energy beyond 10–12 km range.

We interpret this LVZ to be an axial magma chamber (AMC) or zone of melt within the crust beneath the rise axis and use the reflections from the top of this LVZ to infer the width, depth and along-axis variation in the magma chamber.

**Width of the magma chamber.** The results from this experiment provide very tight constraints on the width of the magma chamber at the EPR. The section shown in Fig. 2 appears to be typical of the rise crest south of the Clipperton transform. The reflection from the top of the magma chamber migrates into a relatively flat, high-amplitude event with a maximum width of 2–3 km. We have not yet been able to image either the walls or floor of the magma chamber, but Moho reflections can be confidently traced to within at least 2–3 km of the rise axis on both the eastern and western flanks of the ridge, placing an upper bound of 4–6 km on the total width of any crustal magma chamber along this portion of the EPR. A weak, discontinuous reflector can be traced out onto the ridge flanks at the same depth as the magma chamber reflection that could represent the top of a frozen magma chamber, perhaps marking the contact between the sheeted dykes and the underlying isotropic gabbros. This is the first time, as far as we are aware, that an event such as this has been seen in marine reflection profiles.

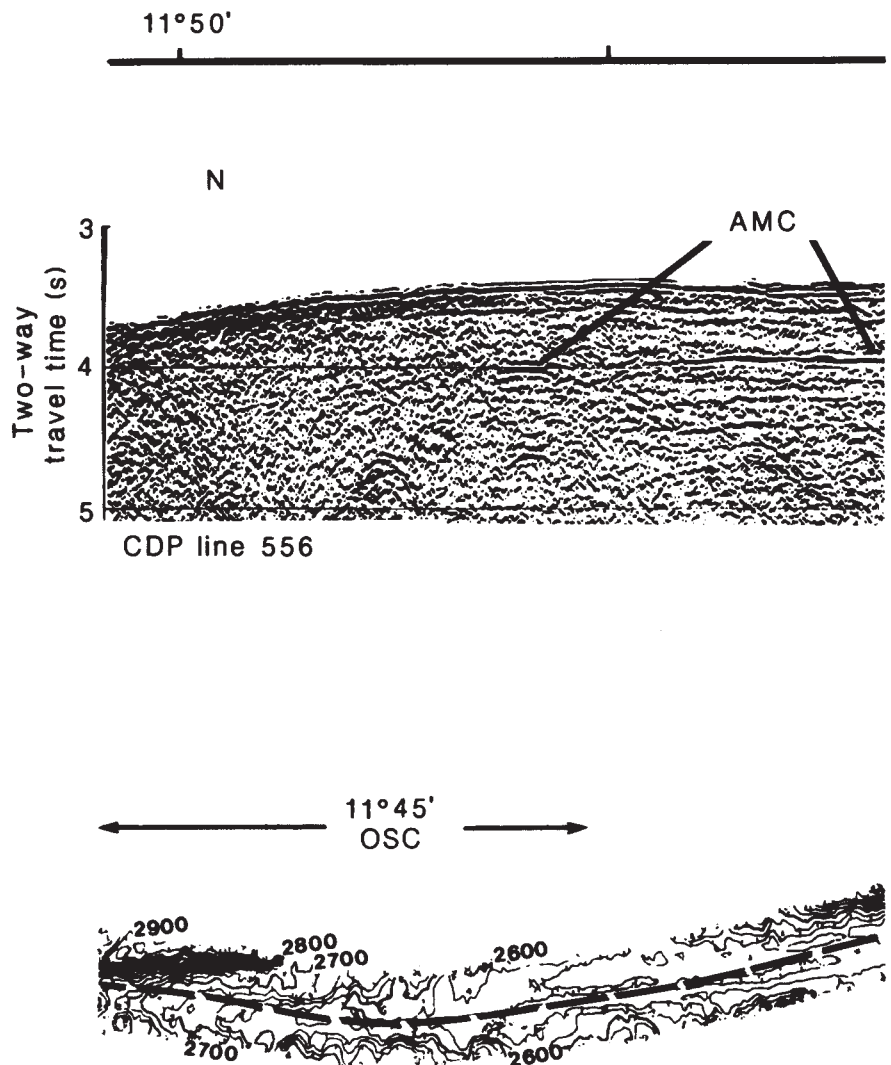
In the 13° N area, a magma chamber reflection is observed

on only a few profiles crossing the ridge, and Moho reflections are generally not seen, possibly due to somewhat rougher ridge-flank topography. Where magma chamber reflections are observed, they appear to be no more than 2 km wide. The existence of a very narrow crustal magma chamber near 13° N is strongly supported by an analysis of the three ESPs shown in Fig. 4. Although a seismic LVZ is present 1.2 km below the sea floor at the rise crest (ESP 9), only 3.6 km away (ESP 13) a typical oceanic velocity structure is present with a well-developed lower crustal (layer 3) refractor and no evidence for a crustal LVZ. Even at ESP 11, 1.5 km from the rise crest, arrivals with phase velocities of 7 km s<sup>-1</sup>, characteristic of the lower crust, are clearly seen beyond 9–10 km range. However, these arrivals are not continuously connected with arrivals at shorter ranges having phase velocities typical of the upper crust (layer 2). The existence of this small shadow zone indicates the presence of a thin (<300 m thick) LVZ sandwiched between the upper and lower crust. However, the total width of the magma chamber in this area is probably less than 4–6 km, and only 1.5 km from the ridge axis the seismic LVZ, if present at all, is extremely thin.

Our estimate of the width of the magma chamber at the EPR is thus well-constrained by the width of the reflections from the roof of the chamber, the gap in the Moho reflection at the rise axis and the crustal velocities from ESPs in the axial region. The narrow magma chamber we have found (4–6 km maximum, generally <3–4 km wide) is incompatible with the large (>10–20 km), funnel-shaped bodies proposed on the basis of some ophiolite models<sup>3,26</sup>, but is consistent with the results of recent refraction experiments at the EPR<sup>17–19</sup>.

**Depth of the magma chamber.** The depth of the top of the magma chamber is also well-constrained in our experiment by the ESPs shot along the rise axis and the two-way travel time between

**Fig. 3** Time section of a 100-km-long segment of CDP line 556 along the EPR south of the 11°45' N OSC illustrating the along-strike continuity of the magma chamber reflection. Lower panel shows a 20-m Sea Beam map of this portion of the EPR crest and the location of the MCS profile (dashed line). The reflection data have been bandpass filtered 5–40 Hz, corrected for spherical divergence, predictively deconvolved (filter length 750 ms; prediction distance 40 ms) and filtered again 6–20 Hz. The data are displayed as in Fig. 2. Deval locations after Langmuir *et al.*<sup>11</sup>; stars indicate areas of reported hydrothermal activity<sup>24</sup>.



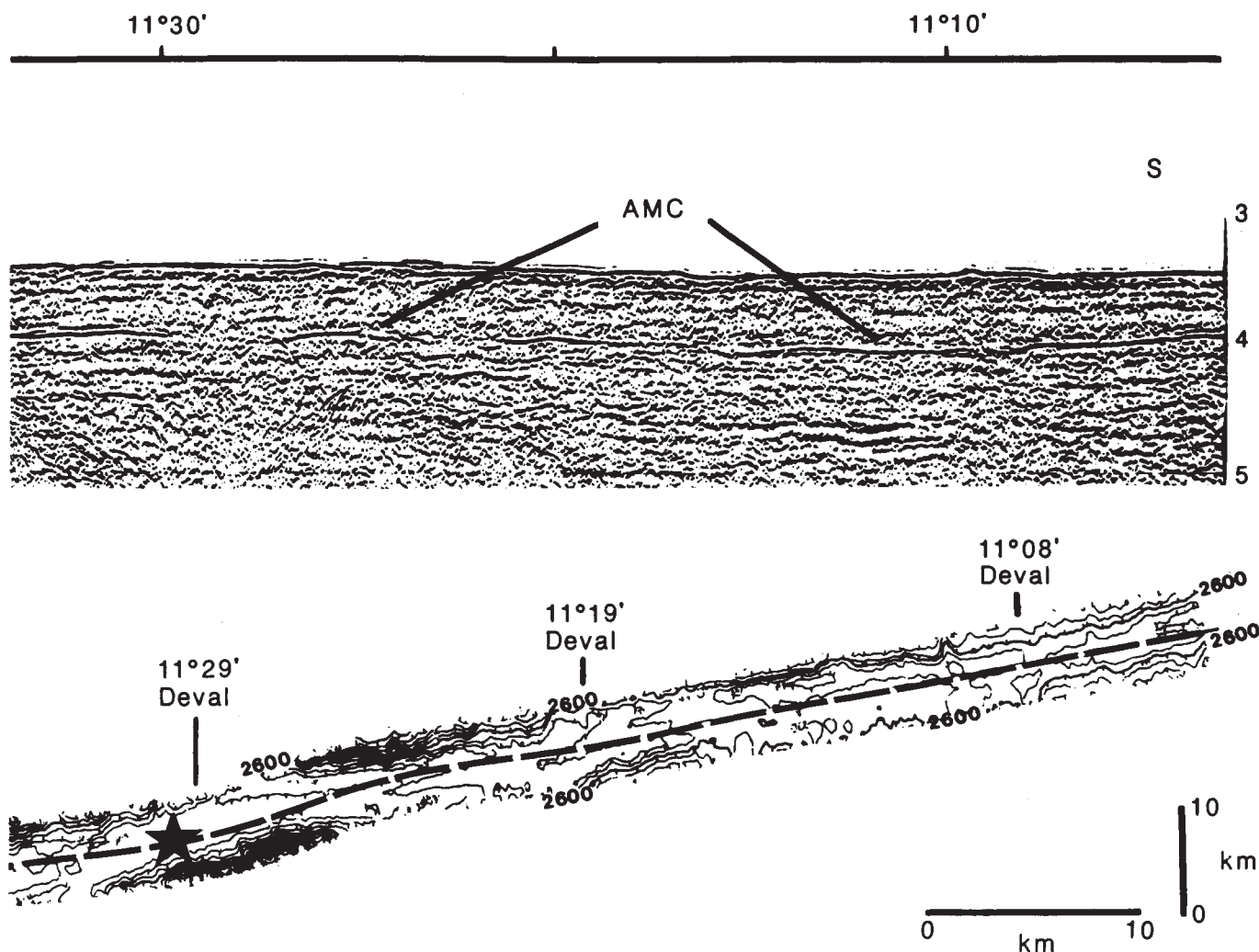
the sea floor and the AMC reflection. Along the portion of the EPR we have surveyed, the roof of the magma chamber varies in depth between 1.2 and 2.4 km below the sea floor, a result which is consistent with previous estimates based on seismic refraction studies at the EPR<sup>15–18</sup>. The most interesting new observation made in this study is the correlation between the depth of the magma chamber (that is, the thickness of the crustal lid above the chamber) and the depth of the rise axis. Although the depth of the EPR varies <300 m along the 500 km of ridge we surveyed, the depth of the magma chamber changes by more than a kilometre. In general, the shallowest parts of the rise axis (south of Clipperton, between 11° N and 11°40' N, north and south of the 12°54' N OSC) are associated with the thinnest crustal lids, while the magma chamber reflection is deeper, more discontinuous and, in some cases, absent altogether where the rise axis is deep (north of Clipperton, near the OSC at 9°03' N and 11°45' N). These results are a striking confirmation of the inferences of MacDonald *et al.*<sup>9</sup> of magmatic budget based on axial geomorphology.

**Along-strike variation in the magma chamber.** As part of this experiment, we obtained a unique series of reflection profiles along the crest of the EPR between 8°50' N and 13°30' N, using Sea Beam to keep the vessel positioned atop the axial high. An AMC reflection is observed along slightly more than half (61%) of the 500 km of ridge crest we surveyed (Fig. 5). The most continuous magma chamber reflection is found south of the Clipperton transform, where a high amplitude event can be traced more than 90 km from just north of the large 9°03' N

OSC to a point about 10 km south of the Clipperton transform (line 571). There are three locations, dashed on the interpretive section, where the AMC event becomes weak and somewhat discontinuous. However, in each instance we believe this was caused by the vessel sliding off the axial high rather than a physical discontinuity in the magma chamber. Several other small gaps in the AMC reflection (for example, 11° N, 11°29' N, 11°40' N, 11°50' N) may have a similar origin. Magma chamber reflections that are laterally continuous for 40–50 km are also observed south of the 12°54' N OSC on line 554 and between 11° N and 11°40' N on line 556. These results are the first geophysical evidence for the continuity of an axial magma chamber over distances of several tens of kilometres at the EPR.

The largest gap in the AMC reflection (~77 km) occurs north of the Clipperton transform. This ridge segment is deep (>2,700 m) and relatively narrow (<4 km wide), plunging over 200 m toward the Clipperton ridge-transform intersection (Fig. 5). The absence of an AMC reflection along this part of the EPR indicates that a magma chamber, if present at all, is much smaller than to the south. Seismic refraction studies had previously suggested the possible absence of a crustal magma chamber in the vicinity of ROSE line 6 near 11°19' N<sup>20</sup>. We observe a clear, phase-reversed AMC event on line 556 in this area (Fig. 3). However, no corresponding event is seen on the cross-axis profiles, again suggesting that the magma chamber is quite small or partially solidified. The presence of a small, seismically unresolvable AMC along a substantial length of the ridge segment north of the Clipperton transform is the strongest





geophysical evidence so far that crustal magma chambers are not steady-state features at these spreading rates.

The AMC reflection deepens toward the large OSCs at 9°03' N and 11°45' N. It can be traced into the region where the spreading centres overlap, but in each case the magma chamber reflections disappear before reaching the tip of the overlapping ridges (Fig. 5). Reflection profiles across and through the overlap basins at both OSCs do not reveal an AMC reflection, suggesting that the axial magma chamber is physically disrupted. These observations, and the small dimensions of the magma chamber documented above, clearly favour the OSC model of MacDonald *et al.*<sup>9,28</sup>. However, both their model and Lonsdale's<sup>29,30</sup> model predict a continuous magma chamber beneath small-offset OSCs. We observe a gap in the AMC reflection across the small OSCs at 12°54' N and 12°37' N (Fig. 5), suggesting that the magma chamber is disrupted as predicted by Langmuir *et al.*<sup>11</sup> on petrological grounds. However, the AMC reflector is generally continuous across many of the smaller ridge axis discontinuities located between OSCs, including seven of the ten Devals identified along this portion of the EPR (Fig. 5). It may be significant that several Devals (9°17' N, 9°53' N, 11°08' N, 12°28' N) are located near or close to prominent dips in the AMC reflection, although in these areas it is difficult to keep a ship located precisely on top of the axial high.

## Discussion

We have not yet been able to image either the walls or floor of the magma chamber, but some aspects of its shape can be

constrained by our data. Figure 6 shows a model that is consistent with our observations. The magma chamber is modelled as a flat-topped body with a roof 2–3 km wide. We include a thin region of liquid melt in the top of the chamber to explain the high amplitude of the AMC reflector and its reversed polarity. However, both of these observations require only a strong, negative velocity contrast and do not necessitate the presence of a liquid. A chamber consisting, for example, of a partially solidified crystal mush<sup>31</sup> would also be consistent with our results. The observation of Moho reflections very close to the rise axis, and our ESP data, indicate that the chamber does not widen with depth but remains quite narrow.

One of the major remaining uncertainties concerns the thickness of the chamber. In Fig. 6 we envision a mushroom-shaped magma chamber with a bulbous shallow top and a narrower central stalk that extends to depths equivalent to Moho in the adjacent crust. We base this model on the assumption that, were the chamber much thinner (for example, a partially molten sill), unreasonable values of the elastic attenuation factor,  $Q$ , would be required to explain the complete extinction of arrivals beyond 11 km range on the ridge axis ESPs. However, we have no direct knowledge of the physical properties of the material within the chamber or its thickness and, consequently, the root structure of the magma chamber shown in Fig. 6 is still largely speculative.

There is a good correlation between some of the geochemical and petrological variations in basalts dredged from this portion of the EPR<sup>11</sup> and our seismic results. The basalts with the most homogeneous compositions and highest eruption temperatures

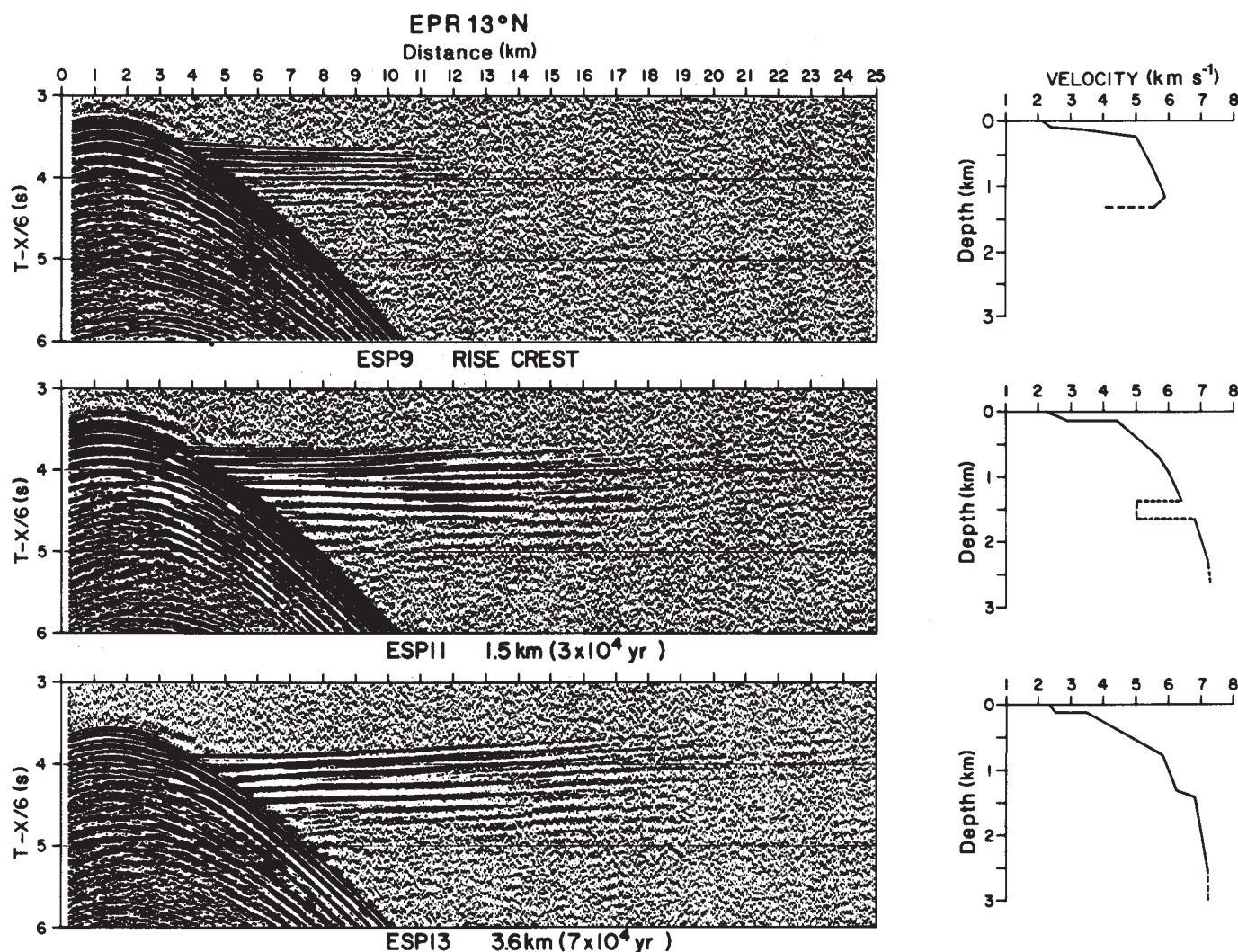


Fig. 4 Record sections for three ESPs shot along and parallel to the EPR near 13° N. ESP 9 (top) was shot along the rise axis, ESP 11 (middle) and ESP 13 (bottom) are 1.5 and 3.6 km from the ridge axis, respectively. The source in each case was a 35.21 (2145 in<sup>3</sup>) airgun array. The data have been gathered into 50-m bins, summed, bandpass filtered 6–40 Hz, and plotted with a reducing velocity of 6 km s<sup>−1</sup> and a gain that is scaled linearly with range. Compressional wave velocity solutions for each ESP are shown on the right derived from travel-time modelling.

were dredged from the ridge segment south of the Clipperton transform where we record some of the strongest, most continuous magma chamber reflections. Low eruption temperatures and more heterogeneous compositions are found in basalts along the ridge segment north of Clipperton where we do not observe a magma chamber event. The gaps we observe in the magma chamber reflection across both large- and small-offset OSCs also appear to substantiate petrological evidence for a lack of magmatic continuity and mixing across OSCs<sup>11</sup>. However, the smaller offsets between OSCs, such as Devals, are generally not associated with breaks in the magma chamber reflection, and we can trace the AMC reflector continuously across Devals that appear to separate regions of distinctly different petrology (for example, 9°53' N, 11°08' N, 12°28' N).

Based on the geochemical variability of basalts erupted along the ridge, Langmuir *et al.*<sup>11</sup> have recently argued for a much smaller scale magmatic segmentation of the EPR than previously proposed<sup>32–34</sup>. Instead of a single, centrally supplied magma chamber underlying each tectonically defined spreading centre cell, they propose that the AMC can be physically discontinuous within a single cell with multiple injection centres distributed along the ridge. We have seismic data on the along-strike continuity of the AMC reflection from three tectonically defined spreading centre cells: 9°03' N to Clipperton, Clipperton to

11°45' N, 11°45' N to Orozco. In each of these cells we can trace the AMC reflection laterally for distances of at least 40–50 km along strike. We believe a physically continuous magma chamber must exist at this scale along at least portions of the EPR. South of Clipperton transform, where morphological<sup>9</sup>, petrological<sup>11</sup> and seismic evidence all indicate a large, robust magma chamber is present, the entire spreading cell (>90 km) appears to be associated with a single, laterally continuous magma chamber. However, in the other two cells, significant gaps in the AMC reflection may indicate that the magma chamber is much more discontinuous along strike, although the absence of an AMC reflection does not necessarily preclude the presence of a magma chamber.

The picture that emerges from both the seismic and petrological data is of a magma chamber that is quite narrow (<4–6 km wide), but continuous for several tens of kilometres along the ridge axis. Where the magmatic budget is high (for example, south of Clipperton), a single magma chamber can extend along an entire, tectonically-defined spreading centre cell. The chamber will be relatively large and well-mixed, and erupting basalts will be geochemically homogeneous. Where the magmatic budget is low (for example, north of Clipperton), the magma chamber is smaller, or absent altogether, incompletely mixed and disrupted by both large and small OSCs. Lavas will be



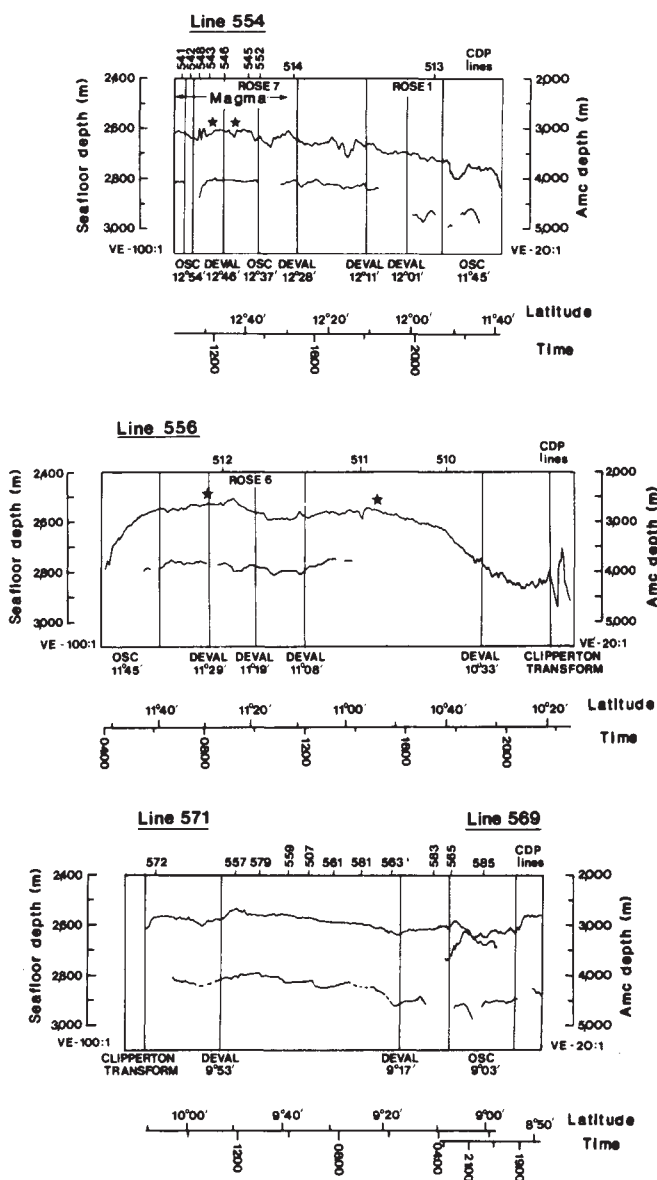


Fig. 5 (Left) Interpretive line drawing of a longitudinal section along the EPR extending from the 9°03' N OSC to the Clipperton Transform (lower panel), from the Clipperton Transform to the 11°45' N OSC (middle panel) and from the 11°45' N OSC to the 12°54' N OSC (upper panel). In each drawing the top curve, plotted using the scale on the left, is the sea floor depth derived from the centre beam of the Sea Beam swath. The lower curve, plotted using the scale on the right, is the depth of the AMC reflection below the sea surface derived from the observed two-way travel time assuming an interval velocity of  $4.9 \text{ km s}^{-1}$  for the crust between the sea floor and this reflector. Each panel is annotated with the location of the ridge crossing profiles (labelled ticks) and various ridge axis discontinuities including transforms, OSC and Devals (vertical bars). The stars mark the locations of known hydrothermal activity<sup>13,24,27</sup>.

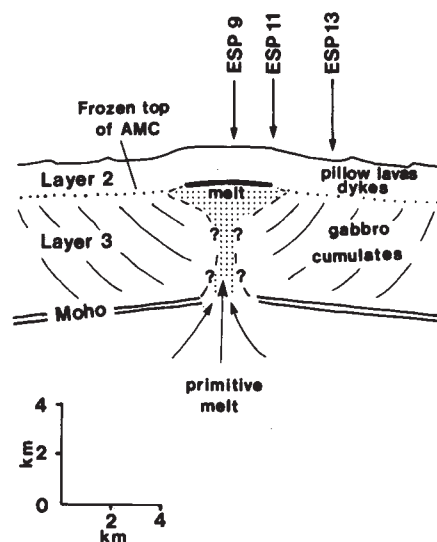


Fig. 6 Schematic drawing of the shape and dimensions of the AMC at the EPR based on the multi-channel seismic results presented in this paper. Location of ESPs shown in Fig. 4 is indicated for comparison. See text for discussion.

geochemically and petrologically diverse. Although smaller offsets between OSCs are not generally associated with physical disruptions in the AMC, geochemical anomalies associated with these features may reflect multiple regions of supply from below the crust or a magma chamber that is incompletely mixed and heterogeneous in its composition. The correlation of this morphological, geochemical and seismic variability along the rise axis appears to offer great promise for an improved understanding of the magmatic and tectonic processes occurring at the EPR.

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# A family of unusually spliced biologically active transcripts encoded by a *Drosophila* clock gene

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*Complementary DNA cloning of the transcripts of the Drosophila clock gene period reveals three distinct transcripts. These result from unusual splicing pathways, one involving a CG 3' splice site and one resulting in the use of two different reading frames in one exon, and they predict three separate proteins. Two of the cloned cDNAs can restore clock function to mutant arrhythmic flies.*

BIOLOGICAL clocks control periodic fluctuations (rhythms) of behaviour and physiological parameters in living organisms. The circadian clock of mammals (for review see ref. 1), which resides in the central nervous system and governs daily fluctuations of various physiological functions and patterns of behaviour, has been extensively studied from a physiological point of view. Molecular genetic aspects of clock function, however, have been studied in more detail in the fly *Drosophila melanogaster*.

The *period* (*per*) gene is one of the four loci in *D. melanogaster* that influence this fly's biological rhythms<sup>2,3</sup>. It is suspected to be directly involved in the oscillators underlying these rhythms for two reasons. (1) Whereas certain *per* mutations abolish rhythms (*per*<sup>0</sup>), others modify their periodicities by shortening them (*per*<sup>s</sup>) or lengthening them (*per*<sup>L</sup>)<sup>2</sup>; this indicates that the gene is not only essential for clock function but is involved in determining period length. (2) The *per* gene influences at least one clock function other than that which underlies circadian rhythms: the male's courtship song rhythm<sup>4</sup>, which has a period length of only one minute and is affected by *per* mutations in a fashion that parallels the way in which they affect circadian rhythmicity.

Recently, genomic DNA from *per* has been cloned<sup>5,6</sup>, introduced back into *per*<sup>0</sup> mutant flies (by means of P-element mediated transformation) and shown to restore their circadian phenotype<sup>7,8</sup>. Analysis of RNA for *per* transcripts has identified a 4.5-kilobase (kb) messenger RNA which is expressed mainly in flies' heads<sup>9</sup>, is absent from RNA of flies with deletions of the *per* locus and is restored after transformation with *per* DNA<sup>8</sup>. DNA sequence analysis and biochemical experiments indicated that *per* codes for a large protein that may have proteoglycan characteristics<sup>10-12</sup>.

To characterize *per* and the putative protein(s) encoded by it further, we have now cloned complementary DNAs for transcripts originating at this locus. We show here that at least three transcripts are encoded by the *per* gene. These transcripts are generated by alternative splicing events, some of which are very unusual. Two of the cDNAs are also shown to be biologically active in restoring the circadian rhythm to mutant (*per*<sup>0</sup>) flies. The structure and possible functions of the putative family of *per* proteins, as well as the unusual splicing events involved in generating them, are discussed.

## Cloning of cDNA

Previous experiments using genomic *per* DNA as a hybridization probe for *Drosophila melanogaster* RNA indicated a major 4.5-kb transcript originating from the *per* locus<sup>5,6</sup>. We expected

therefore to clone a 4.5-kb cDNA which hybridizes to the appropriate band on Northern blots. We constructed two cDNA libraries (see Fig. 1, legend) from which we cloned three types of cDNA corresponding to three species of messenger RNA, all of which appear to be ~4.5-kb long. The level of expression of the two minor mRNA variants (types B and C) is much lower than that of the major type A mRNA (see below). This fact, along with the size similarity of the three types, probably explains why mRNA heterogeneity was not detectable in earlier RNA hybridization experiments<sup>5,6</sup>.

## Type A—the most abundant

The most abundant type of cDNA is designated type A. Its relative abundance is estimated to be about 70%, as six of the eight clones (some of which are shown in Fig. 1b) that extend sufficiently to the 3' end to allow for their classification are of type A. The structure of type A cDNA is shown in Fig. 1b. The entire *per* genomic DNA sequence and the predicted amino-acid sequence of the *per* A protein, as translated from type A cDNA, are presented in Fig. 2.

Jackson *et al.*<sup>10</sup> have recently published a sequence and organizational study of the *per* gene. Of the eight exons described in their study, six are essentially identical in sequence and position to those described here for type A cDNA (our exons 1, 3, 4, 6, 7 and 8 in Fig. 1b). But our exon 2 is absent from their description and our exon 5 is divided into two parts in the summary of their study. The absence of an intron in our exon 5 adds 35 amino acids to the predicted protein sequence, and the addition of exon 2 to the structure extends the N-terminus of our protein by 62 amino acids and places the initiating ATG at position 2,837, as indicated in Fig. 2. Because our analysis of the gene organization is based mainly on cDNA clones which cover the entire transcribed region, we believe that the structure which is presented (Figs 1b and 2; see legend to Fig. 2) is correct.

In summary, we predict for the major type A *per* transcript an RNA of 4,519 nucleotides in length (excluding the poly(A) tail), containing a 368-base pair (bp) untranslated 5' region. The translated exons code for a 1,218 amino-acid protein and there are 497 untranslated nucleotides at the 3' end. The predicted relative molecular mass (*M<sub>r</sub>*) of the protein is 127,000 (127 K), in the absence of any post-translational modification.

Expression of type A mRNA was evaluated by Northern blot analysis using a probe that contains sequences shared by type A and C cDNAs (probe 4, Fig. 1a). (Note that all type A sequences are included in type C; see below and Fig. 1b.) As type A is much more abundant, probe 4 detects mainly the expression of type A mRNA. Figure 3b (lane H, upper band) shows that type A is mainly expressed in adult flies' heads; only very low levels of transcript are observed in body and embryo RNA (see lanes E, B, H). This result agrees with those from

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**Fig. 1** Maps of *per* gene and cDNAs. *a*, Genomic restriction enzyme map of the *per* gene<sup>6</sup>; *b*, maps of *per* cDNAs as obtained in this study. *a*, The genomic probes used for the cDNA cloning experiments and subsequent mapping experiments are shown above the map and are referred to by numbers. A genomic fragment which was used in previous P-element mediated transformation experiments<sup>7</sup> is indicated below the map. Its 3' end extends beyond the right end of the map. Its 5' end, however, does not include the 5' end of the transcription unit of the 4.5-kb mRNAs. The location of the specific oligonucleotide (GGAATCGGAAAGCTCTGTGC) that was used to prime cDNA synthesis from the 5' end of the *per* mRNA is indicated by a dash. Restriction enzymes symbolized as follows: R, *EcoRI*; H, *HindIII*; N, *NcoI*; P, *PstI*; Bg, *BglII*; S, *SacI*; X, *XmnI*; B, *BamHI*; Bs, *BstEII*; Sn, *SnaBI*; Hg, *HgiAI*. *b*, The three proposed structures of *per* cDNAs are presented as types A, B and C. The specific cDNA clones which were analysed in detail to derive these structures are presented below the relevant cDNA maps; their names are in the column on the right. Their borders are defined by vertical bars, unless a cloning artefact such as intramolecular recombination or attachment of irrelevant sequences took place. These events occurred only at the 5' ends of some clones and are represented by wavy lines. The cDNA maps are made of boxes (exons) or lines (introns). Open boxes, coding sequences; black boxes, non-coding sequences. The exons are numbered only in the abundant type A cDNA. CG designates the introns containing non-consensus, CG-mediated, 3' splice sites (referred to in text), and GT the region of the glycine-threonine repeat coded for by *per* cDNAs (see text). Broken arrow indicates the change of the reading frame of type C cDNA as compared to types A and B (see text); asterisk, (type B cDNA) use of a 5' splice junction site 20 bp upstream of the one used by type A for the splicing of exon 1 to exon 2.

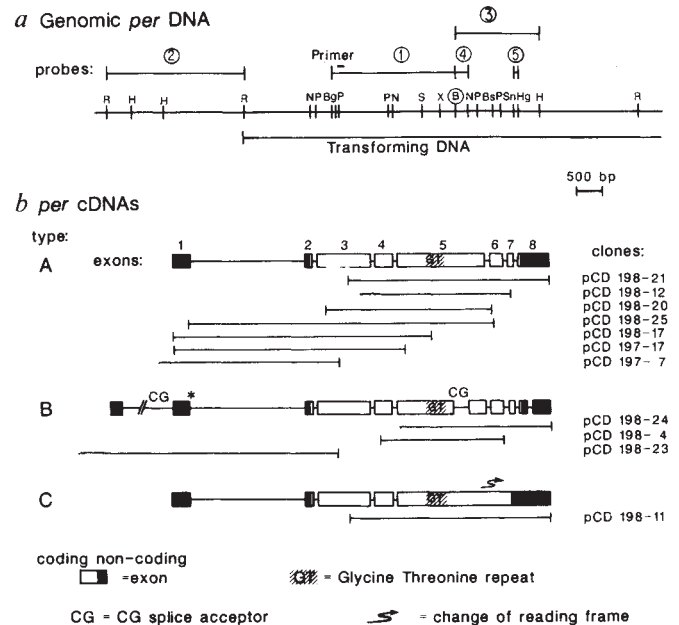
**Methods.** RNA was prepared from heads, bodies, whole flies and embryos of *D. melanogaster* (Canton S strain), essentially as described<sup>36-38</sup>. All the probes used for hybridizations were DNA fragments labelled by the oligo-labelling technique<sup>39</sup>, using random primers from Pharmacia. Probe 5 was a 65-bp *SnaBI*-*HgiAI* fragment from clone pCD198-11 that was subcloned into the *HincII* and *PstI* sites of pSP64. The labelled RNA probe was synthesized using SP6 polymerase from Promega Biotec, following their protocol. Hybridizations were carried out for 24–36 h at 42 °C in 50% formamide, 5×SSCPE (500 mM NaCl, 75 mM Na citrate, 50 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM Na<sub>2</sub> EDTA, pH 7.2), 10×Denhardt's, 0.5 mg ml<sup>-1</sup> herring sperm carrier DNA. When the RNA probe was used, transfer RNA (10 µg ml<sup>-1</sup>) was included in the hybridization mixture. Probes were used at 10<sup>6</sup> c.p.m. ml<sup>-1</sup>. For cDNA cloning, 9 µg of poly(A)<sup>+</sup> RNA, made from heads of *D. melanogaster* strain Canton S, were used as described<sup>40,41</sup> with the following modifications. First strand was synthesized using MLV cloned reverse transcriptase (BRL, 2,000 U per reaction). The reaction was carried out in the buffer described<sup>40</sup>, but included, in addition to oligo(dT), 200 ng of the oligonucleotide primer described above. The reaction mixture was incubated 60 min at 37 °C. Second strand synthesis was as described<sup>40</sup>, except that no ligase was added, and the incubation was 12 h at 12–14 °C after which fresh aliquots of DNA polymerase I and RNase H were added for an additional incubation of 2 h at 22 °C. The cDNA was then blunt-ended, *EcoRI* methylated and linkerized, after which the free linkers were separated from the cDNA on a Sepharose 4B-CL column. To obtain the non-size-selected library, the cDNA was ligated to λgt11 phage arms<sup>42</sup>, which were previously digested with *EcoRI* and dephosphorylated. Another fraction of the cDNA was electrophoresed through a 1% low-melting-temperature agarose gel and the cDNA >1.7 kb in size was extracted and ligated to λgt11 arms to obtain a size-selected library. Both libraries were packaged using Gigapack (Vector Cloning Systems) packaging extracts and were screened before amplification. Probe 1 (see above) was used to screen the size-selected library and 32 positive clones were identified. Probe 2 was used to screen the non-size-selected library and 19 clones were identified. The other probes denoted above were used to analyse and classify the isolated clones.

previous experiments using Northern blots and *in situ* hybridizations<sup>9</sup>, namely, that *per* is mainly expressed in nervous tissue. That this type A 4.5-kb band originates from the *per* locus was confirmed by its absence from RNA of mutant *per*<sup>-</sup> flies which are homozygous for a deletion of the *per* locus (lane D, Fig. 3b). RNA from mutant flies, transformed with wild-type genomic DNA which restores their circadian phenotype (see Fig. 1a for the 'transforming DNA', fragment '14.6' in ref. 7), shows the reappearance of a faint hybridizing band slightly larger than 4.5 kb (lane T, Fig. 3b). An additional band of low *M<sub>r</sub>* is also observed in the lane with RNA from flies' heads. As it also appears in the lane with RNA from *per*<sup>-</sup> flies, this band has not been characterized further.

### Type C—alternative reading frame

One of the cDNA clones (pCD198-11, Fig. 1b), denoted type C, does not splice out the three most 3' introns of type A. Thus it contains a very large exon (of about 2,800 bp) starting at the 5' border of exon 5 of type A and ending at the end of exon 8. The clone extends on its 5' side up into exon 3 where the cDNA ends. On the 5' side of the *BamHI* site (circled in Fig. 1a), this clone contains splices identical to type A (between exons 3 and 4, and 4 and 5; see Fig. 1b). A rough estimate is that the frequency of type C cDNA is about 10% of *per* cDNAs, as only one out of the 8 clones which extend far enough to the 3' to be classified was of this type.

To assess the expression of type C mRNA, we have subcloned probe 5 (Fig. 1a), which hybridizes to type C but not type A or B DNA sequences (see below and data not shown). The use of this fragment to probe Northern blots revealed a band of ~4.5 kb that, like type A mRNA, is expressed mainly in adult flies' heads and is not present in RNA from *per*<sup>-</sup> adults (see upper band in Fig. 3a). The band which hybridizes to the type



C-specific probe is slightly larger than the type A band (compare to Fig. 3b, which is the same blot reprobed with type 'A+C'-specific probe). The small difference in size can be accounted for by the three type A introns, 193 bp altogether, which are not excised in type C. The small size difference also indicates that the 2.3-kb intron (between exons 1 and 2 of type A) cannot be part of the type C mRNA. We assume therefore that the 5' end of this mRNA is identical to that of type A.

Type C cDNA diverges from type A at the end of exon 5 (Figs 1b and 2). Interestingly, the sequence of the last 107 amino acids of the putative type C protein is entirely different from the 149 amino acids at the end of the type A protein. This stems from the fact that the type C mRNA, by reading through the intron which lies between exons 5 and 6 of type A, then reads through the entire exon 6 in a different frame from the one used by type A (Fig. 2).

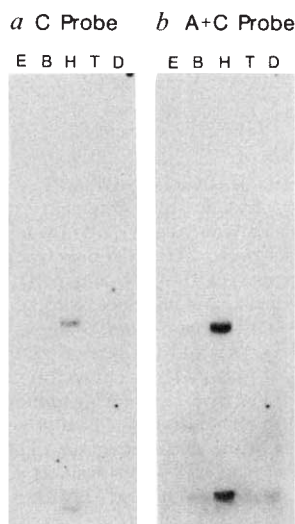
### Type B mRNA uses a non-consensus splice site

Of the 16 cDNA clones that span the *BamHI* site (circled in Fig. 1a), two clones (~10%) did not have this site (pCD198-4 and pCD198-24, Fig. 1b). One of the clones (pCD198-24) ends at the same 3' region as do types A and C. But in this clone exon 8 of type A is split into two exons by an 89-bp intron (see dashed underlines indicating the splice junctions of this intron in the bottom of Fig. 2). This splice does not result in any changes in the putative protein coding sequence, because it occurs downstream of the stop codon shared by type A and B cDNAs. The *BamHI* site is missing from these 2 clones due to yet another splicing event which takes place in exon 5 of type A. In this instance a relatively large intron of 288 bp is excised, resulting in a deletion of 96 amino acids from the predicted type A protein sequence (see Fig. 1b for the CG intron; see Fig. 2 for the predicted type B protein sequence). The 3' splice site

[illegible]

**Fig. 2** DNA sequence of the *per* gene and the predicted amino acid sequence of the putative *per* proteins. The sequence of genomic *per* DNA from *D. melanogaster* (Oregon R strain) was obtained on both strands by the dideoxy method as described.<sup>47,48</sup> The cDNA clones were mapped by comparison to the genomic DNA; in places where they differed, the cDNAs were sequenced according to Maxam and Gilbert<sup>43</sup>. In this way the precise positions of introns were determined; these positions were consistent with those obtained by RNase protection experiments (data not shown). The protein sequences are derived from the conceptual translation of the relevant sequences. Exon 1 is non-coding, and the predicted initiator methionine is present in exon 2. This methionine is followed by a very long open reading frame (in the derived cDNA sequences) and is preceded by an in-frame stop codon. Its surrounding sequences conform well to rules established for translational start sites<sup>44</sup>, especially for *D. melanogaster* (D. Cavener, personal communication). The numbers within the sequence indicate amino acids. The numbers in the right margin indicate nucleotides from the *Hind*III site, which is 178 bp upstream of the beginning of exon 1 (the second H from the left in Fig. 1a). Exon 1 is non-translated and is set off from the upstream nontranscribed sequence as well as from the downstream large intron (intron 1) by shifts in the printed lines. The transcription start is at position 179, as determined by RNase protection mapping (data not shown and ref. 10). In addition, two cDNA clones, pCD198-17 and pCD197-17, extended to positions 192 and 210, respectively. The alternative 5' junction, which is used by clone pCD198-23 (presumably type B cDNA) to join exon 1 to exon 2, is indicated by a broken underline. The possible 3' splice junction of this clone near the 5' end of exon 1 is also indicated by a broken underline, as are all the additional type B-specific splice junctions throughout the sequence. The rest of the splice junctions are shared by type A and B cDNAs and are underlined. All three putative proteins share the first 862 amino acids of the protein sequence. The protein sequences of the three proteins (*per* A, B and C) are indicated by the letters in the right margin which designate which of the *per* proteins share the sequence in the line to the left. As indicated in the text, type C cDNA does not splice out the last three introns of type A cDNA; the resulting alternative reading frame used by type C is shown in the line below the translation of the open reading frame used by types A and B for this sequence. The first letter in the three letter amino acid code is written below the first base of the translated codon triplet. The 3' untranslated sequence present in the cDNA clones is also shown up to the point of poly(A) tail addition. A comparison of the genomic sequence of Oregon R *per* DNA shown with ~4,800 nucleotides of *per* DNA from Canton S<sup>10</sup> reveals only 14 base substitutions. Nine of these are in the coding DNA and all nine are third base isocoding substitutions. Four are in the introns (one in intron 6 and three in intron 7) and one is in the 3' untranslated region. The Canton S sequence<sup>10</sup> lacks the C at position 5,290 in the Oregon R sequence. Also, the Thr-Gly repeat region is 18 nucleotides (or six pairs of Thr-Gly) longer in Canton S<sup>10</sup> than Oregon R; this is a true strain polymorphism and will be the subject of a separate report.





**Fig. 3** Northern blot analysis of type A and C mRNAs. 10  $\mu$ g of poly(A)<sup>+</sup> RNA from embryos (E), bodies (B), heads (H), total flies transformed with *per* genomic DNA (see Fig. 1a for 'transforming DNA') (T), and total flies deficient for *per* DNA, *Df(1)TEM202/Df(1)64j4* (ref. 6) (D), were electrophoresed through a 1% agarose formaldehyde gel and transferred to a membrane filter. (Note that electrophoresis was carried out for a relatively long time to resolve type A and C mRNAs.) *a*, Blot hybridized first to a type C-specific RNA probe (see probe 5, Fig. 1a), then washed (see Fig. 1) and exposed to X-ray film for 4 days. *b*, The same blot rehybridized to the *Bam*HI-*Nco*I DNA fragment (Fig. 1a, probe 4), which hybridizes to both type A and C cDNAs but not to type B. The blot was exposed to film for 24 h. The previous type C-specific signal, which was much fainter than the present one, washed off during the rehybridization.

in this case deviates from the AG rules<sup>13</sup>, using the dinucleotide CG instead. The sequence immediately 5' to the splice site is rich in pyrimidines and agrees well with the rest of the consensus for 3' splice sites (see Fig. 4). The 5' splice site obeys the consensus rules reasonably well (Fig. 4). The CG splice site is present in both Canton S<sup>10</sup> and Oregon R (this study) genomic sequences. Moreover it is present in the DNA sequence of the type A and C cDNAs (which do not use this splice junction). It is also noteworthy that *D. melanogaster* has only one *per* gene from which type B mRNA must be transcribed.

We found evidence in yet a third clone (pCD198-23, Fig. 1b) for a similar event occurring in a different position. In this clone, a new sequence is joined to a point 47 bp downstream of the transcription initiation site of exon 1 of type A (see dashed underline at the 5' end of exon 1 in Fig. 2). As we have not analysed the 5' side of this putative intron, we cannot be certain that it is a true splicing event, that is, one using a consensus 5' splice site. It is interesting, however, that the putative 3' splice site is again a CG rather than an AG dinucleotide. Figure 4 shows the 12 nucleotides immediately 5' to the two non-consensus 3' splice junctions described above. In both cases the pentamer CGYCG (with Y denoting a pyrimidine) precedes the 3' junction.

The splicing event between exons 1 and 2 is also slightly different in clone pCD198-23, where the 5' splice site of exon 1 is 20 bp 5' to that used by the abundant type A (see dashed underline near the 3' end of exon 1 in Fig. 2). It is noteworthy that both of the changes in exon 1 of clone pCD198-23 do not result in protein sequence changes. The putative extra 5' exon of this clone, however, does suggest that the mRNA complementary to it may use a different promoter, one which is 5' to that used by type A and presumably by type C. The fact that a CG 3' splice site may be used in two different positions in two independent and relatively rare *per* cDNA clones raises the

|                | 5' Junction                        | 3' Junction                         |
|----------------|------------------------------------|-------------------------------------|
| Consensus:     | G/GT <sup>A</sup> <sub>8</sub> AGT | (pY)N <sup>G</sup> <sub>7</sub> AG/ |
| pCD198-24 & 4: | G/GTGTGG                           | CTTCTCCTCCGCCG/                     |
| pCD198-23:     | ?                                  | CAACGCGTTCGTG/                      |

**Fig. 4** Non-consensus splice sites in type B cDNA. The 5' and 3' splice site sequences of the CG-mediated splice of type B cDNA (clones pCD198-4 and pCD198-24) are shown below the consensus 5' and 3' splice site sequences<sup>45</sup>. The possible CG-mediated 3' splice site at the 5' end of type B is also shown (clone pCD198-23). As discussed in the text, the 5' splice site of this putative intron is not known; (pY) denotes a polypyrimidine stretch.

possibility that they might originate from the same mRNA. Therefore, in spite of the fact that clones pCD198-24 and pCD198-23 are not linked, we have classified both of them as type B cDNAs (Fig. 1b). But further analysis will be necessary to clarify the relationship of clone pCD198-23 to downstream sequences.

We have tried to assess the expression of type B mRNA and the occurrence of the unusual, CG-mediated splice by RNase protection experiments. We did observe protected bands which are consistent with the unique splices of type B mRNA (not shown); but because of the low abundance of this transcript, other equally intense background bands obscured the results. A Northern blot analysis with a type B-specific probe was not possible because all type B sequences are included in types A and C (Fig. 1b); also, the putative 5'-specific sequence of clone pCD198-23 was not separable from sequences shared with type A.

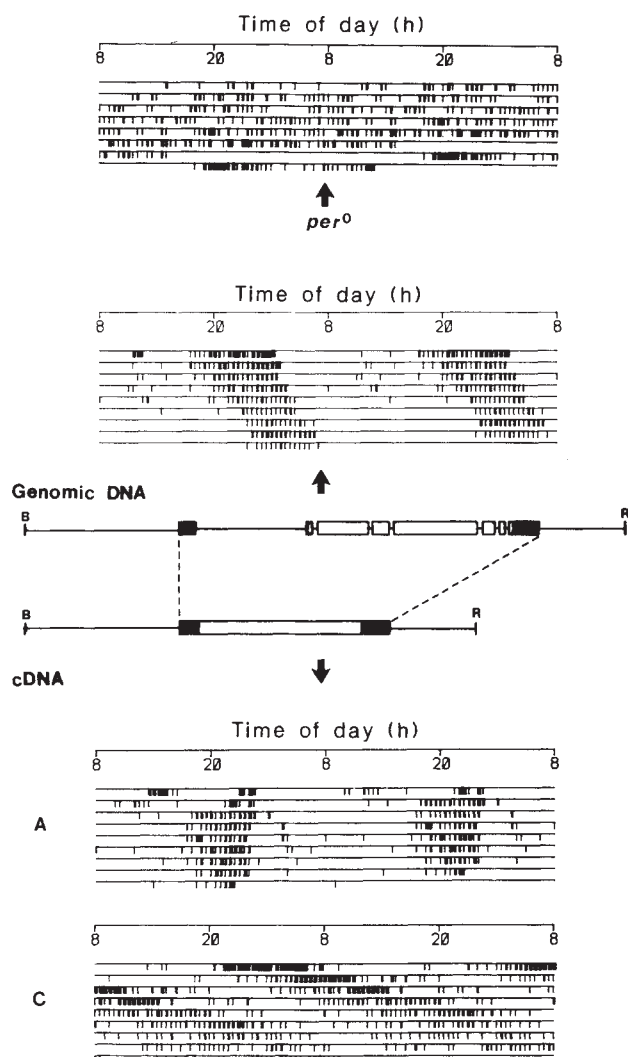
### Unusual splicing events

The use of an alternative reading frame, similar to that described here for type C cDNA, as a means of increasing the coding potential of a gene is unusual in eukaryotes, probably because it renders the gene more sensitive to the effects of mutations. Such a situation has been described for some bacterial as well as mammalian viruses<sup>14,15</sup>, where the rationale would be the need to compact the genetic material. The only other reported evidence for a similar event in eukaryotes (in the case of the human gastrin-releasing peptide prohormone mRNAs<sup>16</sup>) is incomplete because the corresponding genomic sequence is not known.

Although deviations from the 5' splice junction 'GT rule' exist (there are a few reported cases of a GC 5' splice site<sup>17-21</sup>), the 3' splice site 'AG rule' is strictly conserved<sup>13</sup>. To the best of our knowledge, the case described here for type B *per* cDNA is the first to deviate from the AG 3' splice site rule<sup>13</sup>. The presence of a pyrimidine in the -2 position (C instead of A) is especially noteworthy. One possibility to explain this finding is that some novel aspect of the splicing machinery—perhaps associated with the brain or a specific subset thereof—may be necessary to recognize this unusual splice site. Were the second case of a 3' CG junction (in clone pCD198-23) shown to be genuine, the argument for such a novel mechanism would be further strengthened. Consistent with such a mechanism is the fact that three of the four unusual cDNAs show more than one deviation from the major type A structure.

### Intron recruitment

It has been proposed by Gilbert<sup>22</sup> that a single point mutation at a splice junction can contribute to gene evolution by recruiting a whole intron to become part of a coding exon. Perhaps the unusual CG 3' splice site used by type B cDNA was once a consensus and efficient AG 3' splice site preceded by a polypyrimidine stretch (Fig. 4). According to this evolutionary scheme, the 'historically' abundant *per* transcript might have



**Fig. 5** Restoration of circadian rhythms to *per*<sup>0</sup> flies by transformation with the genomic '13.2' fragment and with type A and type C cDNAs. The type A cDNA construct was tailored from clones pCD198-21 and pCD198-25 and type C cDNA was constructed from clones pCD198-11 and pCD198-25, which were ligated to flanking genomic sequences through a unique *Mlu*I site in exon I and the downstream *Hind*III site in exon 8. Type A and C cDNAs (see Fig. 1b), and the genomic DNA which codes for them, were ligated into P-element transformation vectors<sup>46</sup> such that they were flanked by 3.7 kb of 5' genomic sequence (up to the 5' *Bam*HI site, see ref. 6) and by 2.0 kb of genomic sequence at the 3' end (down to the *Eco*RI site; see Fig. 1a). These vectors included the *rosy*<sup>+</sup> marker<sup>46</sup>. Transformants (whose genetic background consisted of *per*<sup>0</sup> and *ry*<sup>506</sup>) were screened for a *ry*<sup>+</sup> phenotype (see ref. 35). Adults from these strains, carrying one dose of a given 'insert'-bearing chromosome (again marked by *ry*<sup>+</sup>), were monitored for locomotor activity under free-running conditions<sup>35</sup>, and the accompanying circadian rhythmicity (or lack of it) was assessed (see below). The activity records shown are for one control *per*<sup>0</sup> (*ry*<sup>506</sup>) segregant from strain '13.2:2' (see Table 1) in the top panel; for a fly transformed with the 13.2 kb of genomic *per* DNA (from strain '13.2:2') in the second panel; for a type A cDNA transformant (strain 'cA:22') in the third panel; and for a type C cDNA transformant (strain 'cC:46') in the bottom panel. The period estimated for the '13.2' fly (from  $\chi^2$  periodogram analysis<sup>33</sup>) was 24.5 h, that for the type A cDNA fly was 24.0 h and that for type C was 28.5 h. The *per*<sup>0</sup> record shown here was statistically arrhythmic. The 13.2-kb genomic fragment is identical to the 12.8-kb fragment used in a previous experiment<sup>35</sup>. We presume that the phenotypic rescue was less successful in these previous experiments for artefactual reasons.

been type B mRNA. A splice junction point mutation (see, for example, ref. 23) could have occurred to change the AG to CG, resulting in a reduction in the efficiency of this 3' splice site (surprisingly not to zero) and a recruitment of the coding potential of the type B intron (labelled as the downstream 'CG' in Fig. 1b) to the new type A *per* mRNA.

Because *per* is a gene that controls complex behavioural phenotypes, it may have undergone more recent evolutionary changes than many other genes; the unusual type B and C mRNAs may then reflect recent events in the history of *per*. Perhaps there has not been sufficient time for further evolution to eliminate or to stabilize these unusual transcripts. It is noteworthy, in this regard, that the mRNAs of the *Drosophila* memory gene *dunce* show marked heterogeneity<sup>24</sup>.

### Types A and C rescue arrhythmic flies

Previous germ-line transformation experiments of mutant arrhythmic (*per*<sup>0</sup>) flies, using genomic DNA from the *per* region, have indicated that this DNA is indeed involved in the biological clock phenotype. But our data (Figs 1 and 2) and those of Jackson *et al.*<sup>10</sup> indicate that none of the genomic fragments used in the early transformation experiments include the entire transcribed sequences of any of the three 4.5-kb transcripts described here. The absence of part of the 4.5-kb transcription unit may explain the incomplete phenotypic rescue obtained in previous studies<sup>7,8</sup>.

To examine the involvement of the 4.5-kb transcripts in the control of clock functions we performed two kinds of transfor-

mation experiments. (1) Transformation of *per*<sup>0</sup> arrhythmic flies with a 13.2-kb genomic DNA, which (we now know) contains the entire transcription unit for, at least, type A and C mRNAs. In addition, this construct contains 3.7 kb of 5' flanking sequences and 2.0 kb of 3' flanking sequences. (2) Transformation of arrhythmic flies with a construct containing type A cDNA and with a construct containing type C cDNA, each containing the same flanking sequences as described above for the genomic fragment. Because we do not as yet have the 5' sequences of type B cDNA, we could not carry out transformation experiments with this cDNA.

Figure 5 shows typical activity profiles (actograms). A *per*<sup>0</sup> control fly (upper panel) shows scattered bursts of activity with no pattern and was arrhythmic by periodogram analysis. A *per*<sup>0</sup> fly transformed with the 13.2-kb genomic DNA fragment (second panel) shows circadian rhythmicity that is as strong as that of flies with endogenous wild-type *per* DNA. The penetrance (the fraction of flies that show the phenotype) of these transformants is also very high (see Table 1). This indicates that the 13.2-kb genomic fragment may contain all the coding and regulatory sequences needed for a full restoration of the clock function. The type A cDNA transformant (third panel) also shows restoration of circadian rhythms similar to that of the 13.2-kb genomic transformant and of wild-type flies. The penetrance of type A transformants, however, is lower than that of the genomic transformants (Table 1). The lower panel of Fig. 5 shows the actogram of a type C cDNA transformant. This fly also showed a rhythmic phenotype, though its period is longer



**Table 1** Transformants carrying genomic DNA or cDNAs from *per*

| Transforming DNA | Transformants                                     | No. rhythmic | Mean period (h $\pm$ s.e.m.) | No. arrhythmic |
|------------------|---------------------------------------------------|--------------|------------------------------|----------------|
| Genomic 13.2 kb  | 13.2:2                                            | 17           | 24.9 $\pm$ 0.1               | 0              |
|                  | 13.2:34                                           | 21           | 24.7 $\pm$ 0.1               | 2              |
| cDNA             | type A                                            | cA:8         | 24.3 $\pm$ 1.3               | 4              |
|                  |                                                   | cA:22        | 23.8 $\pm$ 0.3               | 3              |
|                  | type C                                            | cC:32        | —                            | 3              |
|                  |                                                   | cC:46        | 26.8 $\pm$ 0.8               | 3              |
|                  |                                                   | cC:55        | —                            | 2              |
|                  |                                                   | cC:68        | 24.5                         | 6              |
|                  |                                                   | cC:74        | 25.0                         | 2              |
| Controls         | <i>per</i> <sup>+</sup> / <i>per</i> <sup>o</sup> | 11           | 24.6 $\pm$ 0.2               | 0              |
|                  | <i>per</i> <sup>o</sup>                           | 0            | —                            | 12             |

The 13.2-kb genomic DNA and type A and type C cDNA constructions were generated as described in Fig. 5 and used to produce germ-line transformants. Two independently isolated strains for both the genomic 13.2-kb construct and for the type A cDNA construct, and five for the type C cDNA construct, were isolated and tested for rhythmicity (see Fig. 5). The transformed strains are named in the second column, with arbitrary isolation numbers following the colons. Summaries of the behavioural results are listed in the three other columns. The proportions of significantly rhythmic adults and mean circadian periods ( $\pm$ s.e.m.) from each strain were derived based on the testing and analytical methods cited in Fig. 5. Each transformant carried one 'dose' of a *per* DNA insert-containing autosome. Males from the two '13.2' strains yielded shorter periods ( $24.5 \pm 0.1$  h,  $n = 23$ ) than did females ( $25.1 \pm 0.1$  h,  $n = 15$ ); this implies that the gene's activity in these transformants is dosage compensated<sup>33</sup>, such that one 'dose' of *per*<sup>+</sup> in a male is expressed at a higher level (leading to a shorter period, see below and ref. 34) than the equivalent dosage in a female, even when this X-chromosomal material is transduced to an autosome (see ref. 33) in the '13.2' strains. Controls were thoroughly *per*<sup>o</sup> in genotype, or carried one dose of normal *per* information on one of their X chromosomes (the 11 *per*<sup>o</sup> heterozygotes). The latter genotype routinely leads to periods slightly longer than normal values (which are  $24.0 \pm 0.1$  h, from tests of 23 *per*<sup>+</sup>; *ry*<sup>506</sup> males; refs 34, 35).

than normal as indicated by the slope of the actogram. The penetrance of type C transformants (Table I) is also lower than that of the genomic 13.2 kb transformants. This type C cDNA transformation experiment should be interpreted with caution because type C cDNA could potentially give rise to type A mRNA by further splicing events (see Fig. 1b).

In contrast to the genomic 13.2-kb fragment, the inability of the cDNAs to restore a wild-type-like circadian phenotype, with a period of about one day and essentially all individuals being rhythmic, may be due to one of the following. (1) The absence of regulatory sequences, which may be present in the missing introns (especially the large first intron), may affect the tissue

distribution and levels of expression of the mRNAs. (2) Splicing *per se* may affect the level of mRNA transport to the cytoplasm<sup>13</sup>; thus transformation with the genomic construct may result in higher steady-state levels of mRNA. (3) Unlike the cDNAs, the genomic fragment may have the potential to encode all of the *per* proteins, two or more of which may be necessary to restore a full circadian phenotype. Additional transformation experiments and mRNA expression studies will be needed to sort out these possibilities.

## The *per* proteins

The three cDNA types cloned in this study potentially code for three proteins. Our transformation experiments indicate that at least *per* A and possibly *per* C are involved in clock function. Recent work has molecularly mapped the *per*<sup>o</sup> and *per*<sup>s</sup> mutations to type A's exon 4 and the 5' half of exon 5, respectively (ref. 25 and M. W. Young, personal communication). Because these mutations lie in sequences that are shared by the three cDNAs (Figs 1 and 2) and may affect the function of all three *per* proteins, they do not indicate whether the putative proteins *per* B and C are also clock protein(s).

An attractive possibility is that one *per* protein (probably *per* A) functions in the circadian clock, whereas another one functions in the courtship song clock<sup>4</sup>; a third may function in yet another clock phenotype (see, for example, ref. 26). This possibility is especially interesting because different species of *Drosophila*, although they exhibit the same 24-hr circadian period, differ markedly in their courtship song period length<sup>3,4,27</sup>, indicating a possible divergence in the control of the two clock phenotypes. Moreover, mosaic analysis of *per* mutations has indicated that the circadian clock is located in the fly's head<sup>28</sup>, whereas the courtship song clock seems to reside in the fly's body, probably in the thoracic ganglia<sup>3</sup>. It will be interesting to see whether the precise spatial distribution of type B (as well as type C) mRNA is different from that of type A.

Because the three *per* proteins inferred here differ in only about 10% of their sequence from each other (see Figs 1 and 2), it seems reasonable to assume that they share the proteoglycan properties, especially since they all contain the entire Gly-Thr amino acid repeat<sup>8,10,11</sup>. Proteoglycans have been shown to be involved in many aspects of vertebrate nervous systems<sup>29-32</sup>. Perhaps the multiplicity of putative *per* proteins, described here, indicates that *per* products are not confined to clock phenotypes, but may also participate in other nervous system functions.

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## Cosmic bubbles as remnants from inflation

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It is generally assumed that primordial density perturbations necessary for galaxy formation are adiabatic perturbations with a flat (scale-independent) spectrum formed during inflation<sup>1-4</sup>. It is not clear, however, whether it is possible to create all types of large-scale structures in the Universe (quasars, galaxies, clusters, superclusters, for example) from one type of perturbation with such a simple spectrum. An alternative approach is based on the theory of cosmic strings<sup>5,6</sup>. However, it is difficult to reconcile this approach with the inflationary paradigm<sup>7</sup>. Recently it was shown that inflation may produce not only adiabatic perturbations but also isothermal perturbations<sup>7-10</sup> with the spectra considerably different from the flat spectrum<sup>7,8,10</sup>. Moreover, phase transitions which occur at the last stages of inflation may lead to formation of exponentially large bubbles and domains containing matter with different density<sup>7</sup>. Here we demonstrate that such effects may be responsible for the observed foam-like large-scale structure of the Universe<sup>11</sup>.

Most contemporary theories of galaxy formation adopt the modern cosmological paradigm, which includes three successive stages: the very early inflationary stage ( $t < 10^{-35}$  s), producing a flat Universe, the density parameter,  $\Omega_{\text{tot}} = 1$ , the hot radiation-dominated Friedman stage, and the matter-dominated stage. As a result of gravitational instability during the last stage, the large-scale structure of the Universe arises from small density perturbations of matter.

Observations performed during the past few years demonstrate that galaxies and clusters of galaxies are not distributed randomly but form systems of different scale, the space between these systems being void of galaxies<sup>12,13</sup>.

A recent deep survey of galaxies in a  $6^\circ \times 114^\circ$  strip shows the present of bubbles of diameter 30–100  $H^{-1}$  Mpc ( $H$  is the Hubble constant in units of  $50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ ), completely void of galaxies and having sharp boundaries<sup>11</sup>.

In the following we shall define two phases in space, bubbles constitute phase B, and the space between bubbles phase S (see also ref. 14). All observed structures are located in phase S, but still the space is largely void of galaxies<sup>13</sup>. Galaxies in phase S form systems of at least three scales<sup>13</sup>: groups of scale  $\sim 1 H^{-1}$  Mpc, clusters of galaxies and galaxy chains of scale  $\geq 10 H^{-1}$  Mpc and superclusters (and other inhomogeneities) of scale 50–200  $H^{-1}$  Mpc. Observations of the Local Supercluster demonstrate the absence of both bright and faint galaxies in voids within and around superclusters. The available data suggest that bubbles are also void of dwarf galaxies<sup>14</sup>. In total one can distinguish structures of several types, supercluster-void structures of small, medium and large scales in phase S, and voids in phase B.

Numerical experiments<sup>15,16</sup> with biased galaxy formation demonstrate that supercluster-void structure of small and medium scale (phase S) can be reproduced in a cold dark matter dominated Universe, however, they are unable to explain the presence of empty bubbles with their relatively sharp boundaries (phase B). The fine structure in phase S is not reproduced in the hot dark-matter-dominated Universe. Thus the presence of structure of different scales and large empty bubbles is a serious challenge to theories of galaxy formation. In what follows we will suggest some possible ways to solve this problem in the context of inflationary cosmology (many relevant details can be found also in ref. 7).

In our opinion, the most natural version of the inflationary universe model is the chaotic one<sup>17</sup>. As distinct from the new inflationary universe scenario, the chaotic inflation model can be realized in a wide class of theories of elementary particles under rather natural initial conditions in the very early universe<sup>18-20</sup>. According to the simplest version of this model, inflation is driven by some massive weakly interacting scalar (inflation) field  $\phi$ . During inflation this field slowly decreases from some initial value  $\phi \geq M_P$  where  $M_P = 1.2 \times 10^{19}$  GeV, whereas the universe at the stage of inflation expands  $\exp[(2\pi/M_P^2)\phi^2]$  times<sup>17</sup>. Inflation stops when the field  $\phi$  becomes smaller than  $O(\frac{1}{3}M_P)$ . Large-scale quantum fluctuations of this field lead to adiabatic perturbations of density with an almost scale-independent spectrum<sup>1-4</sup>.

It was widely believed that such perturbations could be generated only during inflation. This is indeed the case in the theories which describe the evolution of the inflation field only<sup>1-4</sup>. However, in realistic theories of elementary particles there exist many other types of scalar fields  $\psi_i$ . Their perturbations, which are also generated during inflation, in some cases lead to isothermal perturbations of a considerable amplitude<sup>7-10</sup>. What is more important is that the spectrum of such perturbations may differ from the flat one, for example, the spectrum may decrease at small (at large) wavelengths<sup>7,8,10</sup> and may have several sharp maxima<sup>7</sup>.

Especially interesting effects may arise due to phase transitions which occur at the last stages of inflation<sup>7</sup>. In the model discussed above, perturbations on a scale of horizon ( $l_h \sim 10^{28}$  cm) are produced when the field  $\phi$  reduces to the value  $\phi_h \sim 3.2 M_P \sim 4 \times 10^{19}$  GeV. Perturbations on the galaxy scale ( $l_g \sim 10^{22}$  cm) are produced at  $\phi \approx \phi_g = 2.8 M_P$ . Let us assume that the field  $\phi$  interacts with the fields  $\psi_i$ , that is,  $\Delta L(\phi, \psi_i) = -\lambda_i \phi^2 \psi_i^2$ . Typical values of  $\lambda_i$  should not be too large,  $\lambda_i \leq 10^{-6}$ . When the field  $\phi$  decreases from  $\phi_h$  to  $\phi_g$  the effective mass squared of the fields  $\psi_i$  is changed approximately by 20%,  $\Delta M_i^2 \sim 2\lambda_i(\phi_h^2 - \phi_g^2) \sim \lambda_i(2 \times 10^{19} \text{ GeV})^2 \leq (2 \times 10^{16} \text{ GeV})^2$ . But this is the scale of grand unification. Such a change of  $\Delta M_i^2$  may lead to phase transitions with generation (or disappearance) of large classical fields  $\psi_i$  (ref. 7). Such phase transitions are completely analogous to the well-known high-temperature phase transitions in gauge theories<sup>21,22</sup>.

Therefore in realistic theories, in which there are very many different types of the fields  $\psi_i$ , one may expect a sequence of different phase transitions which occur at some critical values of the field  $\phi = \phi_i$  when this field decreases from  $\phi_h$  to  $\phi_g$ . If some of these phases transitions are the first order ones, they proceed by formation of bubbles of the fields  $\psi_i$ . The energy density of all fields  $\psi_i$  during inflation remains much smaller than the energy of the inflation field  $\phi$ . Therefore the interior of each bubble continues expanding exponentially, just as before the phase transition. As a result, at the end of inflation the size of each bubble  $l_B$  grows

$$\exp\left(\frac{2\pi}{M_P^2} \phi_i^2\right)$$

times. A typical size of each bubble  $l_B$  after inflation is given by

$$O(M_P^{-1}) \exp\left(\frac{2\pi}{M_P^2} \phi_i^2\right)$$

and for  $\phi_i$  in the range  $\phi_g \leq \phi_i \leq \phi_h$  the typical bubble size lies in the region  $l_g \leq l_B \leq l_h$ . This may give rise to the bubble-like large-scale structure of the universe discussed above<sup>3</sup>.

Note, that if there is only one first-order phase transition during the evolution of the field  $\phi$  from  $\phi_h$  to  $\phi_g$ , then all large-scale bubbles which can be observed now have a single typical scale  $l_B$ , which is

$$\left[ \frac{2\pi}{M_P^2} (\phi_i^2 - \phi_h^2) \right]$$

times smaller than the horizon scale  $l_h \sim 10^{28}$  cm. However, if



many such transitions occur at different values of  $\phi_i$ , the hierarchy of scales appears,

$$l_{B_i}/l_{B_j} \sim \exp \left[ \frac{2\pi}{M_p^2} (\phi_i^2 - \phi_j^2) \right].$$

In a more general case, phase transitions during inflation lead to formation of exponentially large domains (but not exact bubbles) containing matter in different phases. For example if the field  $\psi$  is the Higgs field responsible for the SU(5) symmetry breaking in grand unified theories the universe at an intermediate stage of its evolution may become divided into domains with symmetry breaking SU(3)  $\times$  SU(2)  $\times$  U(1) or SU(4)  $\times$  U(1). Later SU(4)  $\times$  U(1) phase becomes unstable and also returns to the phase SU(3)  $\times$  SU(2)  $\times$  U(1). The behaviour and structure of domains is crucially model dependent. For example baryon density produced inside different domains after inflation may differ by several orders of magnitude. Domains with a smaller free energy density of matter will expand and if they are relatively rare, they become more and more like bubbles (phase B). On the other hand, if the domains of the energetically unfavourable phase are sufficiently rare, they look like exponentially large drops with a gradually decreasing size. The domain contraction is followed by an explosion and formation of a more or less spherical shell of dense matter rapidly moving from the centre of explosion. This process resembles that suggested by Ostriker and Cowie<sup>23</sup>, but it has a different physical origin. In particular, the domain contraction and explosion may occur much earlier than the time of recombination, and the shell may consist of elementary particles of all types rather than just of baryons.

The two-phase structure of the universe may emerge also due to isothermal perturbations generated at the moment of the second-order phase transition during inflation. Such perturbations have a spectrum with a sharp maximum displaced at some exponentially large wavelength<sup>7</sup>. If the relative amplitude of perturbations of the field  $\psi$  with this wavelength is sufficiently large ( $\delta\psi/\psi \sim 0(1)$ ) then it can be shown that the corresponding density perturbations in the universe also have the foam-like structure consisting of bubbles of exponentially large size. In this case, the boundary between the B and S phases might be not very sharp.

Thus, there exist many different possibilities to obtain the observed foam-like structure of the universe due to the phase transitions during inflation. We wish to note that different mechanisms may lead to a somewhat different structure of phases B and S. In some versions of our model baryonic matter is created predominantly in phase S. Density perturbations may exist both in phases B and S, but only in phase S do baryonic matter form luminous objects. Another possibility is that baryonic matter is present everywhere, but dark matter is present only in phase S, and for this reason, density perturbations in phase B are insufficient for galaxy formation. A similar result is true if galaxy formation occurs due to isothermal perturbations of the field  $\psi$ , which may be absent inside bubbles, that is, inside phase B. A detailed observational study of the large-scale structure of the universe may show which of these possibilities (if any) is actually realized. This would be important not only for cosmology, but also for the investigation of the phase structure of the underlying theory of elementary particles.

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## Detection of strong methanol masers towards galactic H II regions

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Recently, the number of transitions as well as the number of sources showing maser emission of interstellar methanol (CH<sub>3</sub>OH) has been increased significantly. Wilson *et al.*<sup>1,2</sup> detected two new K-band lines to be masing towards W3(OH) and possibly NGC7538. Morimoto *et al.*<sup>3</sup> found masers at mm-wavelengths towards Sgr B2, and Menten *et al.*<sup>4</sup> demonstrated that maser action in the J<sub>2</sub>-J<sub>1</sub> series of E-type methanol, first observed towards Orion-KL<sup>5</sup>, is a common phenomenon in numerous molecular clouds. Observations of the 2<sub>0</sub>-3<sub>-1</sub>E transition of methanol have led to the discovery of strong maser action in a number of galactic sources. Towards W3(OH), NGC6334 and NGC7538, these masers have flux densities of order 1,000 Jy and seem to be closely correlated with compact H II regions. The line intensities are comparable to those observed in OH. Towards other sources absorption or a combination of maser emission and absorption is observed.

The observations were carried out in July 1985 and April-May 1986 with the National Radio Astronomy Observatory (NRAO) 140-foot telescope in Green Bank. In July 1985, the data were acquired using a double-beam-switching technique. Total power mode or frequency switching was used during the 1986 observations. System temperatures were typically ~60 K. The beam-width of the telescope is 2.3 arc min at the observing frequency of 12,178.593 MHz. The flux density scale was established by observations of NGC7027, for which a flux density of 6.33 Jy is interpolated using the formula of Baars *et al.*<sup>6</sup>. The typical r.m.s. pointing accuracy is estimated to be 15 arc s. The observations were greatly hindered by interference from communications satellites which operate at this frequency, and weak features were therefore carefully checked for reality.

Table 1 gives a summary of our observations: the 2<sub>0</sub>-3<sub>-1</sub>E line was detected in absorption and/or maser emission towards all but one of the H II region molecular cloud sources observed by us. In some cases (for example, Ori-KL) we observe weak broad lines. Most spectacular are the powerful masers observed towards W3(OH), NGC6334 and NGC7538 (Figs 1-3). Line parameters for these sources are presented in Table 2. From our single dish offset measurements we can put upper limits of 1.2 arc min on the sizes of the 12.1 GHz emission regions, corresponding to lower limits for the line brightness temperature of 1,200 K (W3(OH)), 340 K (NGC7538), and 2,000 K

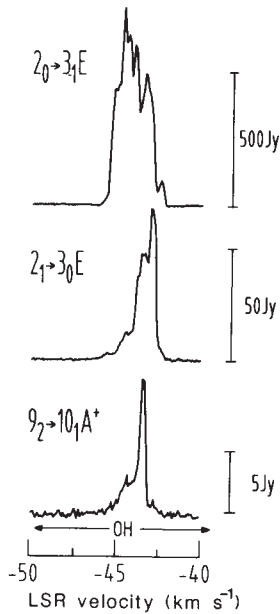


Fig. 1 Methanol masers observed towards W3(OH). The  $2_0-3_1$ E line (velocity resolution:  $0.03 \text{ km s}^{-1}$ ) is shown in comparison to the  $2_1-3_0$ E and  $9_2-10_1$ A<sup>+</sup> lines<sup>1,2</sup> (top to bottom). Note that the emission covers in each case the same velocity interval. The velocity range showing OH maser emission<sup>16</sup> is also indicated.

(NGC6334). Figure 4 shows spectra taken towards W51 as an example of a source where a combination of maser emission and absorption is observed and towards S140 showing pure absorption. From our offset measurements, we find that the absorption towards W51 is clearly extended relative to our beam and appears at higher velocities in the western part of the source, thus showing the same velocity structure as the thermal methanol emission seen in the  $J_2-J_1$  E lines<sup>4</sup>. Unlike W51, S140 has only very weak continuum emission at 12 GHz. It is therefore likely (also in the case of L183) that we are observing here absorption of the 2.7 K microwave background radiation.

Due to its complex level structure, methanol can have a large number of transitions inverted under interstellar conditions. Experience with the observed K-band lines leads to the conclusion that the sources with cm-wavelength methanol masers observed up to now can be divided into (at least) two categories. Firstly, there are the sources (class A) which show maser emission in the  $J_2-J_1$  ( $J=2, 3, \dots$ ) series of E-type methanol. These masers can be found at large offsets (up to 2 pc) from OH and H<sub>2</sub>O masers as well as from compact H II regions and infrared sources which makes it difficult to assign conclusively a powering mechanism to them. Additionally, and perhaps associated with class A masers, broad thermal emission is observed which comes from the compact molecular cores in these regions. Examples include Orion, W31C, W51 and DR21<sup>4</sup>.

Table 1 Summary of measurements

| Source          | Right ascension<br>$\alpha$ (1950) | Declination<br>$\delta$ (1950) | $V_{\text{LSR}}^*$<br>( $\text{km s}^{-1}$ ) | Emission or absorption |
|-----------------|------------------------------------|--------------------------------|----------------------------------------------|------------------------|
| †W3(Cont)       | 02 h 21 min 56.6s                  | 61° 52' 30"                    | -45                                          | A?                     |
| †‡W3(OH)        | 02 23 17.0                         | 61 38 53                       | -45                                          | M                      |
| TMC-1           | 04 38 38.5                         | 25 35 45                       | 6                                            | —                      |
| Ori-KL          | 05 32 46.7                         | -05 24 21                      | 9                                            | E                      |
| §NGC2024        | 05 39 11.4                         | -01 56 09                      | 11                                           | ?                      |
| S255            | 06 09 58.1                         | 18 00 16                       | 17                                           | A?                     |
| S269            | 06 11 46.5                         | 13 50 39                       | 19                                           | M                      |
| IRC + 10216     | 09 45 14.8                         | 13 30 39                       | -26                                          | —                      |
| §Cen A          | 13 22 31.5                         | -42 45 26                      | 546                                          | ?                      |
| L 183           | 15 51 33.8                         | -02 40 30                      | 2                                            | A?                     |
| †¶NGC6334 A     | 17 16 57.8                         | -35 51 45                      | -11                                          | A                      |
| ¶NGC6334 B      | 17 17 00.5                         | -35 49 49                      | -11                                          | —                      |
| ¶NGC6334 C      | 17 17 11.3                         | -35 48 25                      | -11                                          | —                      |
| ¶NGC6334 D      | 17 17 23.0                         | -35 46 20                      | -11                                          | —                      |
| ¶NGC6334 E      | 17 17 29.8                         | -35 43 08                      | -11                                          | M                      |
| †¶NGC6334 F     | 17 17 32.3                         | -35 44 04                      | -11                                          | M                      |
| Sgr A           | 17 42 27.8                         | -29 03 59                      | 15                                           | A                      |
| †Sgr B2         | 17 44 10.5                         | -28 21 58                      | 60                                           | M/A                    |
| †Sgr B2 (south) | 17 44 10.5                         | -28 23 14                      | 60                                           | M/A                    |
| †W31            | 18 07 30.5                         | -19 56 29                      | 0                                            | M/A/E?                 |
| W33             | 18 11 17.9                         | -17 56 45                      | 33                                           | A                      |
| †W49N           | 19 07 49.5                         | 09 01 15                       | 14                                           | M/A/E                  |
| †W51            | 19 21 23.8                         | 14 24 52                       | 60                                           | M/A                    |
| K3-50           | 19 59 50.0                         | 33 24 18                       | -20                                          | —                      |
| †DR21           | 20 37 13.9                         | 42 08 54                       | 5                                            | A                      |
| DR21(OH)        | 20 37 14.8                         | 42 12 08                       | 5                                            | A                      |
| S140            | 22 17 41.1                         | 63 03 40                       | -6                                           | A                      |
| †NGC7538        | 23 11 36.5                         | 61 11 47                       | -58                                          | M                      |
| Cas A           | 23 21 10.0                         | 58 32 24                       | 0                                            | —                      |

\* Centre of observed velocity range, LSR (local standard of rest).

† Additional measurements at offsets (in arc s) of  $(\Delta\alpha, \Delta\delta) = (-75, 0), (75, 0), (0, -75), (0, 75)$  relative to the reference position.

‡ Offsets of  $(-150, 0), (150, 0), (0, -150), (0, 150)$ .

§ Observations impossible due to satellite interference.

|| Observations marred by satellite interference.

¶ For the NGC6334 positions the nomenclature of Rodriguez *et al.*<sup>8</sup> was used.

Second, one finds CH<sub>3</sub>OH maser sources (class B) close to compact H II regions of which the prototype is W3(OH). This source (as well as NGC7538) shows absorption (and thermal emission) in the  $J_2-J_1$  E lines. Towards W3(OH) the  $2_1-3_0$ E and  $9_2-10_1$ A<sup>+</sup> lines have been found to be masing<sup>1,2</sup>. A Very Large Array (VLA) study of the latter transition has shown that the maser emission emerges from unresolved spots (in a 2 arc s beam) coincident with the compact continuum emission and in the same area as the OH masers<sup>7</sup>. The  $2_1-3_0$ E and  $2_0-3_1$ E emission components cover exactly the same velocity interval as the  $9_2-10_1$ A<sup>+</sup> line (see Fig. 1) and thus it is reasonable to assume that all three masers form in the same region. Taking the upper limit on the source size obtained from the VLA observations of the  $9_2-10_1$ A<sup>+</sup> line (1 arc s), we calculate brightness temperatures of  $5-6 \times 10^6$  K for the stronger components of the  $2_0-3_1$  E maser.

Emission from NGC7538 in the  $2_1-3_0$ E and  $9_2-10_1$ A<sup>+</sup> lines is roughly an order of magnitude weaker than in the case of W3(OH) which makes interferometer observations difficult.

Table 2 Properties of the 'strong'  $2_0-3_1$ E methanol masers

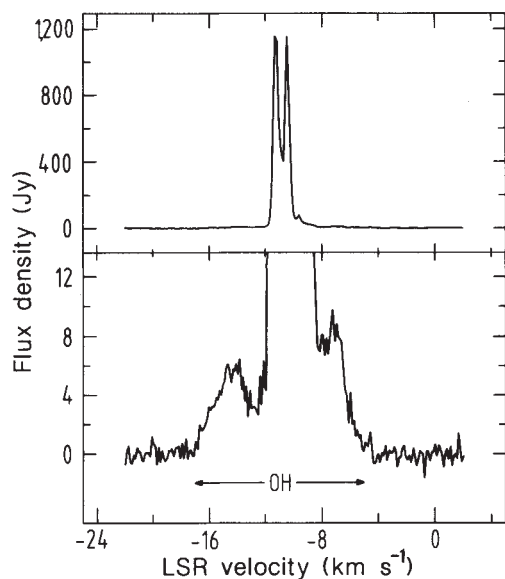
| Source    | Velocity range<br>( $\text{km s}^{-1}$ ) | $v_{\text{LSR}}^*$<br>( $\text{km s}^{-1}$ )  | $\Delta v^*$<br>( $\text{km s}^{-1}$ ) | $\int S dv^\dagger$<br>( $\text{Jy km s}^{-1}$ ) | $L^\ddagger$<br>(photons $\text{s}^{-1}$ ) |
|-----------|------------------------------------------|-----------------------------------------------|----------------------------------------|--------------------------------------------------|--------------------------------------------|
| W3(OH)    | $[-42.5, -46.1]$                         | 10 components in velocity interval            |                                        | 1,297                                            | $3.8 \times 10^{45}$                       |
| NGC6334 F | $[-17, -4]$                              | -11.22(0.01)<br>-10.46(0.01)                  | 0.43(0.01)<br>0.46(0.01)               | 1,147                                            | $2.0 \times 10^{45}$                       |
|           |                                          | several other components in velocity interval |                                        |                                                  |                                            |
| NGC7538   | $[-62, -55]$                             | $\sim -61$<br>-56.39(0.01)                    | $\sim 1$<br>0.82(0.01)                 | 198                                              | $1.5 \times 10^{45}$                       |

\* LSR velocities and linewidths of strongest components are given explicitly only for cases in which fitting of a single gaussian was possible.

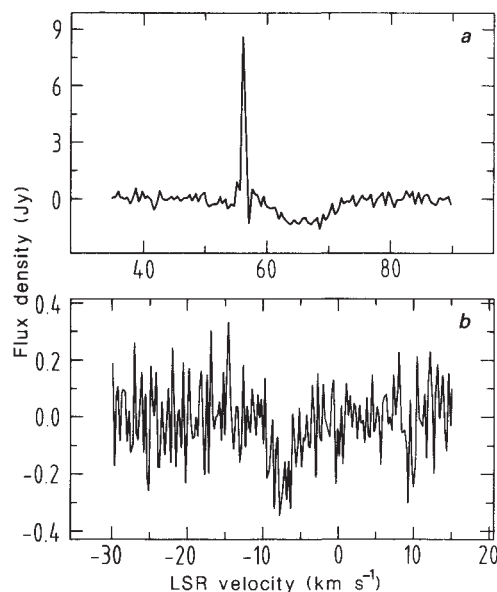
† Flux density integrated over the entire velocity range showing maser emission.

‡ For the calculation of the isotropic photon luminosity  $L$ , we assumed distances of 2.2 kpc (W3(OH)), 1.7 kpc (NGC6334) and 3.5 kpc (NGC7538).





**Fig. 2** The  $2_0-3_1$ E line towards NGC6334 observed with a velocity resolution of  $0.12 \text{ km s}^{-1}$ . The full velocity extent of the emission is best seen in the lower part of the figure where the intensity scale has been zoomed by a factor of 100. The velocity range showing OH maser emission<sup>17</sup> is also indicated.

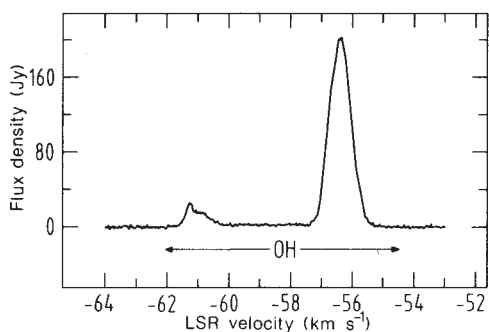


**Fig. 4** Absorption plus maser emission towards W51 (a), and pure absorption towards S140 (b), showing that the  $2_0-3_1$ E line is not in all cases inverted. The velocity resolutions are  $0.48 \text{ km s}^{-1}$  in a and  $0.24 \text{ km s}^{-1}$  (S140) in b.

Thus there is no straightforward way to decide whether these lines are really masing, although this is suggested by the narrow linewidths observed. The high flux density and narrow linewidth ( $0.8 \text{ km s}^{-1}$ ) of the  $2_0-3_1$ E line can only be explained by maser emission and our offset measurements are consistent with a source position coincident with the compact H II region (IRS1).

Towards NGC6334 no  $\text{CH}_3\text{OH}$  lines other than the  $2_0-3_1$ E have been reported hitherto. Our offset measurements reveal, that the strongest features (at  $-11.2$  and  $-10.5 \text{ km s}^{-1}$ ) emerge from positions which are, within the errors ( $15 \text{ arc s}$ ), coincident with the ultracompact H II region<sup>8</sup>, NGC6334 F and the FIR source I of McBreen *et al.*<sup>9</sup>. They also definitely show that the position of the  $\sim 70 \text{ Jy}$  feature at  $-9.6 \text{ km s}^{-1}$  is displaced relative to the strongest features by  $\sim 1 \text{ arc min}$  to the north, thus suggesting a relationship to the more extended continuum source E of Rodriguez *et al.*<sup>8</sup>. We observed absorption towards their continuum source A.

Far infrared radiation has been suggested as a pumping mechanism for OH<sup>10</sup> as well as for  $\text{CH}_3\text{OH}$ <sup>2</sup>. Menten *et al.*<sup>11</sup> find that the physical conditions prevailing in OH maser sources (especially W3(OH)) also favour the inversion of methanol transitions. It is therefore interesting to investigate the observational evidence for a correlation between both species.



**Fig. 3** The  $2_0-3_1$  line towards NGC7538. Velocity resolution is  $0.03 \text{ km s}^{-1}$ . The velocity range showing OH maser emission<sup>13</sup> is indicated.

A first hint of such a correlation is the overlap in the radial velocity ranges indicated in Figs 1–3. One should note that the velocity spread of the  $1,665 \text{ MHz}$  OH masers shown may be an overestimate due to the Zeeman effect<sup>12</sup>. Except for W3(OH), no conclusive information about spatial coincidence of  $\text{CH}_3\text{OH}$  masers with OH masers is available. Obviously, interferometer measurements are needed for the methanol lines. In NGC7538 (ref. 13) as well as in NGC6334 F (Raimond and Eliasson<sup>14</sup> (their source A), Rodriguez *et al.*<sup>8</sup>), the OH interferometer positions lie within the large single dish error boxes obtained from our  $2_0-3_1$ E observations. It is unclear from the literature whether OH emission is present towards NGC6334 E.

Finally, it should be noted that, in the case of W3(OH), the photon luminosity in the  $\text{CH}_3\text{OH}$   $2_0-3_1$ E line is higher by a factor of  $\sim 2$  than the luminosity in the OH  $1,665 \text{ MHz}$  line<sup>10</sup>. This suggests that the methanol abundance is comparable to that of OH and that the pump mechanism is at least as efficient. In W3(OH), the estimated OH abundance is  $>10^{-5}$  (ref. 15), and Menten *et al.*<sup>7</sup> find a  $\text{CH}_3\text{OH}/\text{H}_2$  ratio of  $\sim 10^{-5}$ . Understanding such large methanol abundances is a problem almost as challenging as understanding the maser pump.

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# Evidence for a larger Sun with a slower rotation during the seventeenth century

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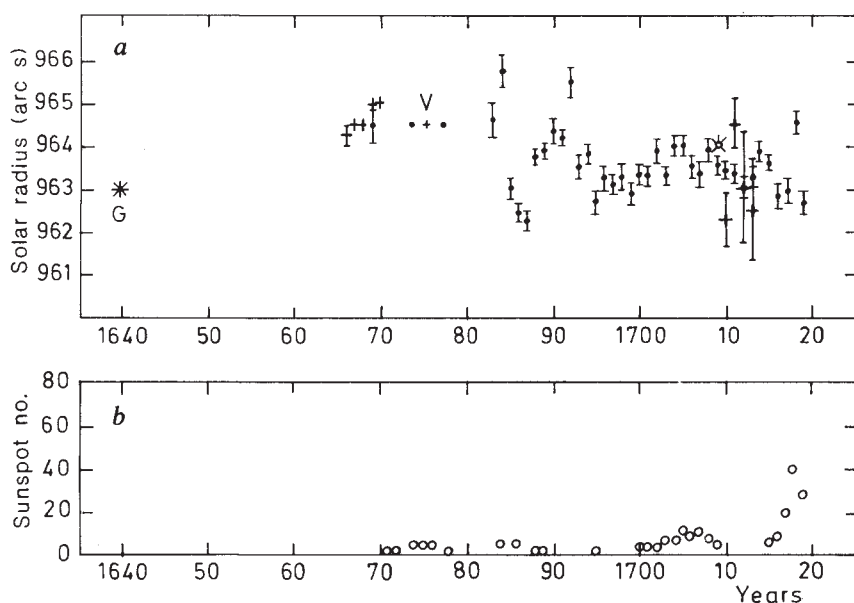
Historical records suggest that during the seventeenth century the Sun was subject to an anomalous scarcity of sunspots (the Maunder minimum<sup>1</sup>). This minimum was coincident with a severe temperature dip of the Little Ice Age<sup>2</sup>, from which a relation between solar activity and terrestrial climate has been conjectured<sup>3</sup>. Solar variability is also suggested by the regular variations in the thickness of annual laminae or 'varves' in the late Precambrian formation in south Australia<sup>4</sup>. The climatic cycles recorded by the varves include the present 10–12-year solar cycle as well as deep minima, with a mean period of 314 years. A natural explanation of the solar cycle climate connection would be a modulation of the solar output radiation, and possibly a change of the solar diameter<sup>5,6</sup>. By analysing a unique 53-year record of regular observations of the solar diameter and sunspot positions during the seventeenth century, we have shown for the first time that the solar diameter was larger and rotation slower during the Maunder minimum. This finding sheds new light on the dynamo mechanism, solar variability, and the consequent effects on terrestrial climate.

J. Picard was the leader of the new school of precision astronomy<sup>7</sup>. He made regular observations of the Sun with a 9½-foot quadrant and 6-foot sextant, both fitted with telescopic sights<sup>8</sup>. He also took advantage of the invention of the filar micrometer (40,000 divisions per foot, better than 0.01 mm.<sup>9,10</sup>), which led to a potential accuracy of 1 arc s (1") in solar diameter determinations (the focal length of the refractor was 6 feet, and the focal solar image was about 2 cm in diameter). In contrast with Hevelius who used plain sight instruments and made qualitative drawings of the solar image, Picard obtained quantitative measurements<sup>11</sup> of the solar diameter, in the meridian plane (method 1), from 1666 up to his death in 1682. He also recorded the transit time of the solar image formed by a meridian refractor (denoted hereafter as method 2). The latter method was extensively used by his colleagues (Ph. and G. de La Hire) from 1683 up to 1719<sup>12</sup>. During each meridian transit, they measured the

transit time of the prior and posterior limb of the Sun, as well as the sunspots. The transit time was recorded with an accuracy of 0.5 s, giving an error of 7" in the solar diameter. With a fixed meridian quadrant, they also measured the height of the upper limb and the sunspot, above the horizon, within ±5". It was then possible for us to reconstruct the position of individual sunspots on the solar disk, and to deduce the solar rotation. The Sun's position and orientation have been calculated from the formulae provided by the Bureau des Longitudes<sup>13</sup>. Subsequently, the position of a sunspot is expressed in the usual heliographic coordinate system.

Apparent horizontal solar diameters obtained from methods 1 and 2 have been calculated, and reduced to the astronomical unit, in order to correct for seasonal variations. Yearly averages of diameters are listed in Table 1 (see also Fig. 1). We distinguish two periods, one corresponding to the deep Maunder minimum (dMm), the second period related to the end of Maunder minimum (eMm), by which time sunspot activity had resumed. The first method was used at the time of dMm and later, from time to time as a check. It gives a mean diameter of 32'9" (that is 964'5" for the radius) during the dMm (see also ref. 14). Such an estimate is obtained as a mean value of 297 observing days, with a r.m.s. deviation  $\sigma = 0.19''$ . So, the statistical error on the mean (s.e.m.) is  $\sigma/\sqrt{297} = 0.011$ . The second method gives a solar diameter of 32'9" over 48 days, with a  $\sigma$  of 4" and a statistical error on the mean of 0.6". For the period 1683–1718, the second method was more often used: the number of observing days per year ranged from 136 to 231 days, and the standard error of the yearly average diameter never exceeds 0.47" (Table 1). During this period, the diameter shows a regular decrease down to 32'6" (that is 963" for the radius). The values of the diameter observed by La Hire at the end of the Maunder minimum are in agreement with measurements by Gascoigne<sup>10</sup> from 1638 to 1641, just prior to the Maunder minimum, and by Tondu<sup>15</sup> during the eighteenth century.

How significant are these findings? As we mentioned earlier, the accuracy of the micrometer itself should not account for more than 1" systematic error on method 1. Spherical and chromatic aberrations could also affect method 1, but this effect is unlikely to have been more than 1", bearing in mind the quality of seventeenth century optics<sup>16</sup>. On the other hand, the second method which relies on transit times is little affected by the geometry of the instrument. It is more sensitive to the personal bias, however, but according to Parkinson *et al.*<sup>17</sup> this bias does not exceed 2.2" for the horizontal diameter. The other important factor is the determination of the transit time: precision of



**Fig. 1** *a*, Yearly averages of the solar radius during the Maunder minimum. Crosses refer to method 1 (using a filar micrometer), dots refer to the transit observations. The  $\times$  symbol is the diameter measured with the filar micrometer at the time of an eclipse. G symbol corresponds to observations by Gascoigne. V refers to Villard's observations. *b*, Sunspot number along the Maunder minimum. Absence of dots means that no sunspot has been detected by the French observers.



**Table 1** Horizontal solar diameter measured by Picard (method 1) and La Hire (method 2) during the seventeenth century

| Year | Observing days | Solar radius (arcs) by method 1 |        |                   | Observing days | Solar radius (arcs) by method 2 |        |                   |
|------|----------------|---------------------------------|--------|-------------------|----------------|---------------------------------|--------|-------------------|
|      |                | yearly average                  | s.e.m. | corrected average |                | yearly average                  | s.e.m. | corrected average |
| 1666 | 38             | 964.25                          | 0.15   | 962.70            |                |                                 |        |                   |
| 1667 | 118            | 964.5                           | 0.09   | 962.95            |                |                                 |        |                   |
| 1668 | 69             | 964.5                           | 0.06   |                   |                |                                 |        |                   |
| 1669 | 61             | 965.02                          | 0.13   | 963.47            | 48             | 964.50                          | 0.43   | 962.73            |
| 1670 | 11             | 965.05                          | 0.15   | 963.50            |                |                                 |        |                   |
| 1671 | dmM            |                                 |        |                   |                |                                 |        |                   |
| 1674 |                | 964.5                           |        |                   | 110            | 964.50                          |        |                   |
| 1676 |                |                                 |        |                   |                |                                 |        |                   |
| 1683 |                |                                 |        |                   | 136            | 964.58                          | 0.47   | 962.81            |
| 1684 |                |                                 |        |                   | 186            | 965.75                          | 0.38   | 963.98            |
| 1685 |                |                                 |        |                   | 162            | 962.98                          | 0.24   | 961.21            |
| 1686 |                |                                 |        |                   | 190            | 962.45                          | 0.16   | 960.68            |
| 1687 |                |                                 |        |                   | 187            | 962.25                          | 0.23   | 960.48            |
| 1688 |                |                                 |        |                   | 207            | 963.75                          | 0.20   | 961.98            |
| 1689 |                |                                 |        |                   | 189            | 963.88                          | 0.22   | 962.11            |
| 1690 |                |                                 |        |                   | 170            | 964.40                          | 0.31   | 962.63            |
| 1691 |                |                                 |        |                   | 154            | 964.20                          | 0.30   | 962.43            |
| 1692 |                |                                 |        |                   | 154            | 965.50                          | 0.37   | 963.73            |
| 1693 |                |                                 |        |                   | 178            | 963.55                          | 0.29   | 961.78            |
| 1694 |                |                                 |        |                   | 186            | 963.90                          | 0.25   | 962.13            |
| 1695 |                |                                 |        |                   | 156            | 962.70                          | 0.30   | 960.93            |
| 1696 |                |                                 |        |                   | 177            | 963.25                          | 0.30   | 961.48            |
| 1697 |                |                                 |        |                   | 181            | 963.13                          | 0.26   | 961.37            |
| 1698 |                |                                 |        |                   | 177            | 963.29                          | 0.30   | 961.52            |
| 1699 |                |                                 |        |                   | 209            | 962.90                          | 0.25   | 961.13            |
| 1700 |                |                                 |        |                   | 187            | 963.36                          | 0.25   | 961.59            |
| 1701 |                |                                 |        |                   | 226            | 963.28                          | 0.24   | 961.51            |
| 1702 |                |                                 |        |                   | 205            | 963.90                          | 0.29   | 962.13            |
| 1703 |                |                                 |        |                   | 202            | 963.33                          | 0.21   | 961.56            |
| 1704 |                |                                 |        |                   | 209            | 963.96                          | 0.24   | 962.19            |
| 1705 |                |                                 |        |                   | 227            | 963.93                          | 0.25   | 962.16            |
| 1706 |                |                                 |        |                   | 210            | 963.49                          | 0.27   | 961.72            |
| 1707 |                |                                 |        |                   | 203            | 963.35                          | 0.28   | 961.58            |
| 1708 |                |                                 |        |                   | 210            | 963.88                          | 0.24   | 962.11            |
| 1709 |                |                                 |        |                   | 202            | 963.55                          | 0.21   | 961.78            |
| 1710 | 14             | 962.25                          | 0.67   | 960.70            | 218            | 963.43                          | 0.24   | 961.66            |
| 1711 | 25             | 964.5                           | 0.60   | 962.95            | 209            | 963.35                          | 0.21   | 961.58            |
| 1712 | 15             | 963.0                           | 1.29   | 961.45            | 213            | 963.00                          | 0.24   | 961.23            |
| 1713 | 13             | 962.5                           | 1.24   | 960.95            | 202            | 963.40                          | 0.23   | 961.63            |
| 1714 |                |                                 |        |                   | 212            | 963.87                          | 0.22   | 962.10            |
| 1715 |                |                                 |        |                   | 230            | 963.55                          | 0.17   | 961.78            |
| 1716 |                |                                 |        |                   | 212            | 962.78                          | 0.26   | 961.01            |
| 1717 |                |                                 |        |                   | 229            | 962.95                          | 0.24   | 961.18            |
| 1718 |                |                                 |        |                   | 175            | 964.53                          | 0.26   | 962.76            |
| 1719 |                |                                 |        |                   | 34             | 962.59                          | 0.25   | 960.82            |

Values are yearly averages and the corresponding standard error of the mean. The corrected average column gives solar radii corrected for irradiation and a second order refraction effect (in the case of transit observations). For the period 1674–76, solar diameters were derived by Villiard (an assistant of Picard), using mainly the transit timings. The results<sup>14</sup> are similar to those obtained by Picard during the period 1666–71.

\* In 1718, an assistant observed for about 4 months, and his measurements are systematically greater than those by La Hire.

clocks was 1 s per day, so, the corresponding uncertainty would be 0.01". In fact, the real source of error is the quantization of time by the observer, about 0.5 s on a mean transit time of 2 min, that is about 7". This uncertainty is quite consistent with the observed r.m.s. deviation. In our reduction procedure, we have used the calculated declination of the Sun, thus eliminating the first-order refraction effect. When the observations are made at a latitude different from 90°, another correction should be applied, according to Wittman<sup>18</sup>. So, the yearly transit radii plotted in Fig. 1 should be decreased by about 0.25". Both methods give a systematic increase of the solar diameter due to irradiation, which has been estimated empirically<sup>19</sup> to be 3.1". Table 1 includes corrected radii. One should remember however, that this irradiation correction is somewhat uncertain and could be larger with the seventeenth century optics.

Observation of the 1715 solar eclipse by Halley has been interpreted<sup>17,20</sup> as leading to a diameter of about 32'0", consistent with the modern value. An eclipse observation is intrinsically more accurate: it is less affected by the personal bias and it measures the difference between the apparent solar and lunar diameters. The departure from the corrected 1715 La Hire observations is 3.5", greater than the 2.2" uncertainty expected

from personal bias. On the other hand, the 1715 eclipse is the only observation available, and its accuracy is strongly dependent on the precise location of the observers. Therefore, the two independent observations (La Hire data and the solar eclipse data) are not incompatible.

There remains the clear 3" decrease of the solar diameter measured by the same skilled observer from 1683 to 1718, which is more than six times the standard deviation of the mean. The variation of the solar diameter, during the seventeenth century reaches  $2 \times 10^{-3}$ , that is three times larger than the uncertainty of the solar limb definition. For comparison, the change of the solar diameter over the 11-yr cycle<sup>21</sup> is only  $2 \times 10^{-4}$ .

An immediate question which arises is the following: does the variation of the Sun's diameter correspond to a real mass motion of the surface layers? The answer can be given from the study of the solar rotation.

As suggested by Maunder<sup>7</sup>, the solar cycle continued to operate at a low activity level. During the 53 yr of observations examined by our Paris colleagues, enough sunspots were sighted so that 11-yr cycles could be reconstructed, maxima occurring in 1674, 1684, 1695, 1705 and 1716. The sunspot activity was concentrated in the southern hemisphere, except for the last

cycle when the sunspots appeared in the northern hemisphere as well.

The longitude of each sunspot is calculated, assuming a rigid rotation of the Sun (a Carrington period of 27.275 days has been used). If the sunspot rotates faster (slower) than the Carrington velocity, the longitude of a given sunspot should increase (decrease) during its transit across the solar disk by an amount proportional to its excess (deficiency) velocity.

For each sunspot, we obtained the variation of longitude during its transit and we made a least mean square linear fit, the slope of which gives the mean acceleration of deceleration of the sunspot with respect to the Carrington velocity. Because of the large uncertainty in the transit time of the spot (7"), there is a large spread in the values of longitude, so we have selected only spots (~100) which could be followed for several days. In this way, we obtain a more reliable estimate of the rotational rate. Since there are few sunspots, there is no ambiguity in identifying them. A drawing of sunspots is enclosed with each observational sheet, which helps to remove any ambiguity. A few return spots have been identified: for these sunspots, the accuracy of the rotational rate is substantially increased and rule out any systematic error coming from an overestimate of the solar diameter. Synodic rotational rates of sunspots have been calculated, as well as a mean rate  $R$  for each 5° latitude belt between -25° and 20°. We also plotted rotational rates by Balthasar and Wohl<sup>22</sup> in the period (1940-1980), and those of Hevelius calculated independently by Eddy *et al.*<sup>23</sup> and by Abarbanell and Wohl<sup>24</sup>, over 1642 to 1644. The bars show estimates of the standard deviation  $\sigma/\sqrt{N}$  for the mean rotation rate for each latitude belt.

Two features are evident in the rotation profile of La Hire's data (Fig. 2). During the Maunder minimum, sunspots rotate slower than their modern counterparts, by as much as 0.5° per day. Furthermore, the differential rotation is greater in La Hire's data than it is today. A very striking feature appears: the rotation profile in the Hevelius data calculated by Eddy *et al.*<sup>23</sup> is very similar to that of La Hire's data. The main difference is the shift velocity (0.8° per day). The difference could be real since the epoch of observations was not the same. An argument in favour of the fast velocity found on Hevelius data was suggested by dynamo theories based on the stretching of field lines by the subsurface radial gradient of angular velocity in the convection zone<sup>25</sup>. One class of models requires an angular velocity increasing inwards. If the high surface equatorial angular velocity arose through a redistribution of angular momentum from deeper convective layers, then the angular velocity at these deep layers should be less than for current solar cycles, thus reducing the angular velocity gradient, and possibly the strength of the dynamo action. However, observed rotational splitting of global oscillations indicates that the angular velocity does not vary much with depth<sup>26</sup>. Also, the discovery of a large-scale meridional circulation of convective origin<sup>27,28</sup> with unexpected properties questions the classical theories of the differential rotation and dynamo. A plausible alternative to the discrepancy mentioned above, as suggested by Eddy *et al.*<sup>23</sup> could be that Hevelius made the disk circle too small, keeping the spots on original scale. The same drawings have been observed independently by Abarbanell and Wohl<sup>24</sup> who found no significant difference with modern data. Cassini<sup>29</sup> also analysed observations of Hevelius, and some earlier data obtained by Scheiner when the Sun was still active. He noticed that the sunspots were, then, rotating with a synodic period ranging from 27 to 30 days, which conforms with the present differential rotation of sunspots. In contrast, his own observations during the 'dMm' showed a slower rotation. Once more, one should emphasize that Hevelius's drawings are qualitative, while La Hire's observations are quantitative. A larger Sun and slower rotation are quite consistent with a real pulsating phenomenon (dilation and contraction of the convective layers), over a timescale of centuries.

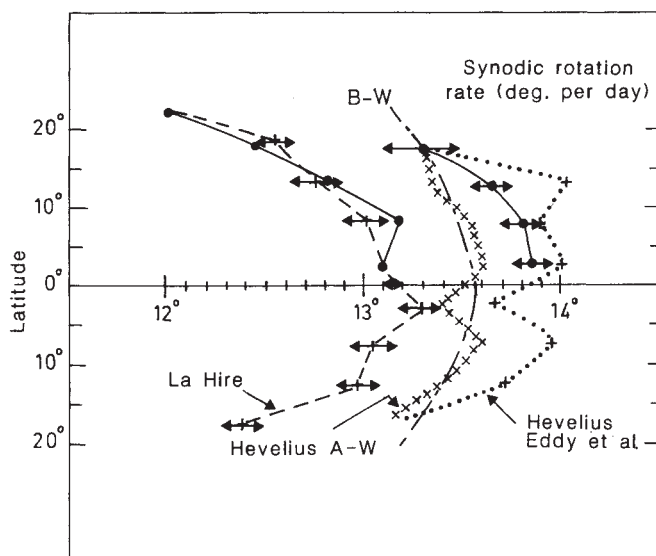


Fig. 2 Rotational rate of one hundred sunspots deduced from La Hire observations during the Maunder minimum: dashed line represents the rotational rate of sunspots in each hemisphere, while solid line is the combination of data. Dotted line and crossed line correspond to the Hevelius data (1642-1644) examined independently by Eddy *et al.*<sup>23</sup> and Abarbanell and Wohl<sup>24</sup>. For comparison, the present rotational curve is also shown (Balthasar *et al.*<sup>22</sup>).

The data presented here reveal that several authors have analysed historical data covering the eighteenth and nineteenth centuries and looked for secular trend in the solar diameter. Their conclusions are diverging, from no trend<sup>17,30</sup> to a small secular decrease of 0.2"<sup>31,18</sup>. None of their data extend beyond 1715 and our data cover a large fraction of the Maunder minimum, so it is not surprising to find a larger effect during this anomalous period. With the wealth of historical data collected during the seventeenth century, we have shown for the first time, that the Sun's diameter was larger than it is now. The departure from the present value (3" at least, during the 'dMm', equivalent to 2,000 km) exceeds the possible systematic errors. On the other hand, we found that the rotational rate derived from the sunspots motion across the solar disk is significantly smaller than the present surface rotation, with an enhanced differential slippage. These two phenomena indicate that there was a real expansion of the solar envelope during the Maunder minimum, suggesting a periodic modulation of the convective zone on a timescale of several centuries<sup>32</sup>. Such an expansion is obviously related to the scarcity of sunspots observed during the seventeenth century. The expansion and contraction of the Sun also seem to follow an 11-yr period<sup>21</sup>. If they are confirmed, a new picture of the solar variability emerges: we have two distinct but strongly coupled cycles: a magnetic 11-yr (sunspot) cycle and an 11-yr convective cycle. They vary in phase; however, the amplitude of their variations is modulated over a longer period (possibly 314 yr). The fluctuations are greater on the long period than on the 11-yr timescale. We note that the solar diameter variation (2,000 km) is 20 times larger than the fluctuation of 150 km detected on the 11-yr cycle<sup>21,31</sup>.

The connection between sunspot activity, solar luminosity and diameter has been suggested by Spiegel and Weiss<sup>6</sup>. If the dynamo source operates in the deep convective layers, the presence of strong fields may alter locally the adiabatic temperature gradient, modifying the convective flux transport. Resulting changes in thermal energy are large enough to produce a variation of the solar diameter and the surface effective temperature ( $T_e$ ). If the energy changes were stored as gravitational potential energy, a decrease in flux through the convection zone would



reduce the radius<sup>31</sup> and we would obtain the radius change as observed. A luminosity variation of 1% could be obtained, and this would account for the cold weather experienced in Europe during the seventeenth century<sup>33</sup>.

Alteration of the convection will also affect the rotation of the solar envelope. We already know that surface differential rotation changes along the solar cycle (torsional oscillations observed by Howard *et al.*<sup>34,35</sup>). This is explained by the presence of counter-rotating azimuthal rolls<sup>27</sup>, in the convective zone. During their movement from low to high latitudes, they accelerate or decelerate latitude belts by transport of angular momentum, causing a flexion in the rotation profile<sup>28</sup>. The differential rotation is greater during the minima of the 11-yr cycle, when the rolls appear to be absent. During the Maunder minimum, the differential rotation was also greater, with a low activity level. Therefore, we suggest that the large-scale convection which is responsible for the magnetic flux emergence was also absent, in relation with the Little Ice Age. Through the alteration of the rotation, the large-scale convective circulation probably plays a major role in the energy transport and the modulation of the output radiation<sup>36</sup>.

A deceleration of 0.5% (with respect to the present value) detected on the Greenwich white-light photographic plates<sup>22</sup> occurred at the beginning of the twentieth century. The deceleration coincides with two cycles of low sunspot number. It would be interesting to see whether there was any corresponding change in the luminosity, through a variation of the solar diameter. If so, the convection would exhibit all the periods found in the sunspot number, including the 80-yr period, known as the Gleissberg cycle. Since the same periods are reflected in the Elatina series formed some 680 Myr ago, the modulation of the convection seems to be a permanent feature of the solar cycle. The azimuthal rolls (and related torsional oscillations) are probably the key for understanding the dynamo mechanism and the possible climatic response to solar variations.

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## Ground-based infrared observations of comet Halley

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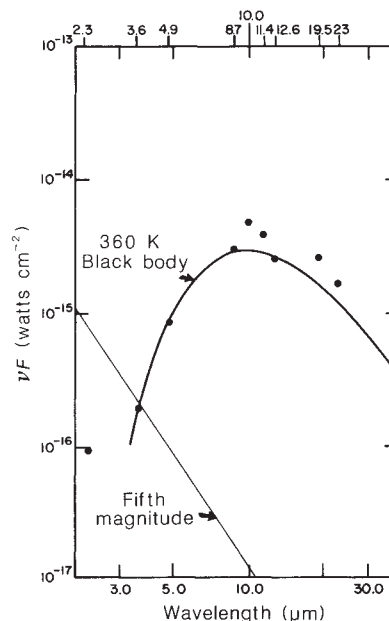
The images of comet Halley's nucleus returned during three spacecraft encounters in March 1986<sup>1,2</sup> are challenging the theory of the release of particles from a comet's nucleus. Instead of a spherically symmetric outflow, each spacecraft observed strong jets flowing from the sunward side of the nucleus. Here we present ground based 2–23  $\mu\text{m}$  photometry and 10.3  $\mu\text{m}$  imaging of Halley taken within hours of the Giotto spacecraft encounter. The photometry shows a colour temperature of 360 K and silicate emission features at 10 and 20  $\mu\text{m}$ , and the image shows jet activity similar to that observed by Giotto, but on a scale of thousands of km. The expected 10  $\mu\text{m}$  surface brightness, based on the particle mass distribution measured by Giotto and assuming solid, spherical grains, is a factor of six lower than the observed value. We suggest that fluffy particles could remove this discrepancy.

We observed comet Halley from 13:00 to 17:00 UT on the morning of March 13 1986 using the 2.3-m telescope of the Wyoming Infrared Observatory (WIRO). Our detector was a single-channel Wyoming Ga:Ge bolometer<sup>3,4</sup> with an a.c.-coupled preamplifier. All observations were made using a 7.5 Hz chopping frequency. On 13 March, Halley was 0.89 AU from the sun and 0.97 AU from earth, giving an image scale of 1 arc s = 703 km.

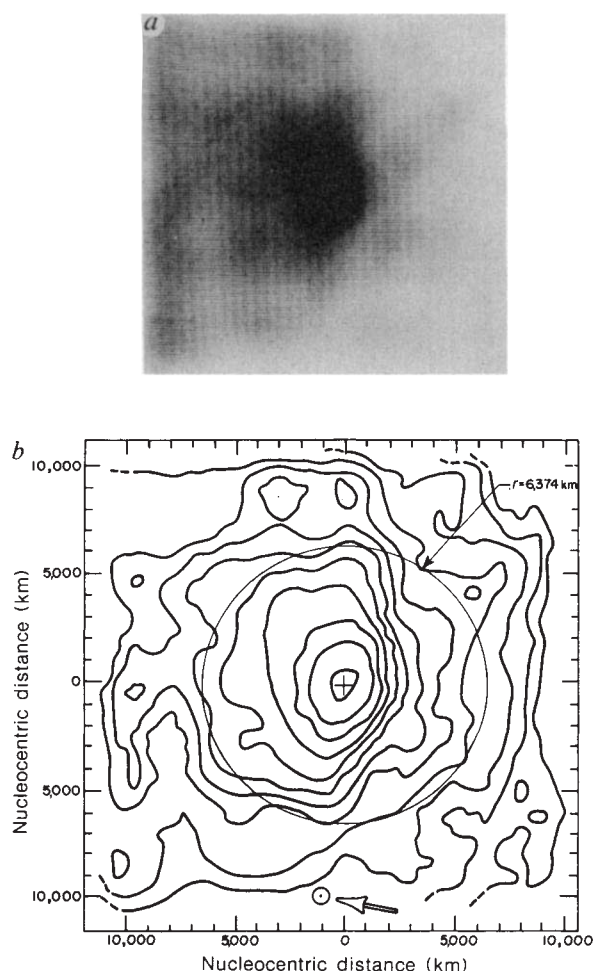
For the photometry (Fig. 1) we used a 5 arc s FWHM (full width at half maximum) circular diaphragm centred on the nucleus, a 20" throw, and standard beam-switching techniques. Alpha Lyrae and Beta Pegasi were the calibrators. These data

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**Fig. 1** Broadband 2.3–23  $\mu\text{m}$  infrared energy distribution of comet Halley's nucleus on 13 March 1986. Error bars are smaller than the plotting symbols. A 360 K black-body curve has been fitted to the data. Emission features at 10 and 20  $\mu\text{m}$  are due to silicates.



**Fig. 2** *a*, Narrow band  $10.3\ \mu\text{m}$  filter image of comet Halley as mapped by the Wyoming Infrared Telescope at 16:56 UT on 13 March 1986. The image is  $32\ \text{arc s}$  ( $22,500\ \text{km}$ ) square, with  $1\ \text{arc s} = 703\ \text{km}$ . A broad fan, possibly composed of several jets, is visible pointing in the sunward direction (toward upper left). *b*, A contour-map version of the same image. Contours are drawn at 10,000, 5,672, 3,781, 2,836, 2,269, 1,891, 1,620, 1,418, 1,134, 945, 810 and  $708\ \text{mJ arc s}^{-2}$ . Except for the  $10,000\ \text{mJ arc s}^{-2}$  contour, these are at intervals of  $11,343/n$ ,  $n = 2, 3, \dots, 8, 10, 12, 14, 16$ , where  $11,343\ \text{mJ arc s}^{-2}$  is the peak surface brightness at the centre of the image. Equally-spaced contours therefore indicate a  $1/r$  surface brightness law. (Note the omission of every other contour beginning at  $n = 8$ ).

are part of an extensive collection of Halley photometry taken at WIRO which is discussed elsewhere<sup>5</sup>.

Two images of the comet and Beta Pegasi were made with a narrow-band  $10.3\ \mu\text{m}$  filter and the WIRO fast mapping technique<sup>6</sup>. The pixel size was  $1\ \text{arc s}$ , the diaphragm size was  $5\ \text{arc s}$ , and the throw was  $40\ \text{arc s}$ , placing the reference beam  $40\ \text{arc s}$  north of the source beam. About four minutes were required to build up one of these  $32 \times 32$  pixel images. The tracking error was less than  $1\ \text{arc s}$  and the seeing was less than  $2\ \text{arc s}$  over this time.

A typical raw  $10\ \mu\text{m}$  image made with our technique shows a north-south intensity gradient due to changes in the detector's line of sight through the telescope as the secondary scans in declination. East-west gradients are not a problem because the entire telescope is driven to cover this dimension. Normally the resulting offsets and north-south gradients are easily determined using blank sky at the edges of the image<sup>6</sup>. With our Halley images, however, the edges are deep inside the coma, so an

additional gradient due to the comet's emission in both the source and reference beams is also present.

To account for these two effects, we first subtracted the mean telescope background seen in the calibration images from the two comet images. By assuming a  $1/r$  surface brightness law for the inner coma, we could then calculate from the raw surface brightness at each pixel (which is the difference between the brightness at that point and a point  $40\ \text{arc s}$  to the north), a value for the surface brightness  $S_0$  at the nucleus.

We calculated  $S_0$  for several pixels in the westernmost four columns of the two images, an area with a smoothly varying surface brightness because it is well away from any jet activity. The mean  $S_0$  was  $13 \pm 1\ \text{Jy arc s}^{-2}$  for pixels in the southern two-thirds of the image, but the  $S_0$  values calculated from pixels near the north edge are twice as high. Other models which included offsets and linear slopes along with the  $1/r$  variation produced worse results. The mean  $S_0$  value for the southern pixels was used to calculate a single north-south baseline correction for each image that would set the westernmost four columns to the proper  $1/r$  profile. The east-west variation in the ideal baseline to subtract from each north-south column is less than 20% of the surface brightness in the four columns we used.

We consider this simple model to be adequate to first order. The larger values of  $S_0$  near the northern edge are probably a result of the very low surface brightness of the comet in the reference beam and errors in the correction for telescope emission. However, it could be due to non- $1/r$  surface brightness variations in the northern half of the image. Our data do not permit us to make more definitive statements.

After adjusting the background, the two images were registered and co-added. The resultant image was sharpened using the maximum entropy statistical method used at WIRO<sup>7,8</sup>. We present the final image in Fig. 2*a* and *b*. The peak central surface brightness of this image is in good agreement with the value of  $S_0$  given above, a further indication that our simple model is reasonable.

The individual raw  $10\ \mu\text{m}$  images already show a broad fan extending from the sunward side of the nucleus. The processed image shown here indicates that this fan is composed of several jets, the brightest of which points  $35^\circ$  east of north and is visible out to about  $3,000\ \text{km}$ . The angle between lines of sight ( $44^\circ$  about an axis through the ecliptic poles) and the rotation of the nucleus in the seven hours between the two measurements ( $48^\circ$  assuming a period of 52.5 hours<sup>9</sup>) must be taken into account when comparing the WIRO image to those of Giotto. Based on the spin-axis orientation deduced from observations<sup>9</sup> in 1910, the dust jets seen by Giotto would have been well within view from earth at the time of our observations.

Our photometric and imaging data, when combined with the *in situ* measurements of Giotto, allow us to estimate some properties of the dust grains in Halley's coma. The observed colour temperature of the continuum emission is  $360\ \text{K}$  (Fig. 1), a factor of 1.22 over the black-body temperature at a solar distance of  $0.89\ \text{AU}$ . Assuming that the mean absorption efficiency for the coma dust grains  $Q_a \approx 1$  across the solar spectrum, the superheat requires a grain emission efficiency of  $Q_e = 0.45$  for the grains producing the continuum emission<sup>10</sup>. If the absorbing grains are graphite, those a few micrometres in size are the dominant radiators in the infrared<sup>11</sup>.

We now estimate the expected surface brightness at  $10\ \mu\text{m}$  from the particle mass distribution observed by Giotto. The three Halley flyby spacecraft found several classes of dust particles, including particles rich in light elements as well as those with a CI carbonaceous chondrite composition<sup>12,13</sup>, but for simplicity we assume most of the mass is in the form of silicate grains. The volume luminosity of such grains is

$$L_v = \int_0^M N(m)mE_m dm$$

where  $m$  is the grain mass,  $M$  is the maximum grain mass,  $N(m)$  is the number of grains per unit volume per unit mass,



and  $E_m$  is the luminosity per unit mass. We use

$$N(m) = 2.40 \times 10^{-6} m^{-5/3} \text{ g}^{-1} \text{ cm}^{-3}.$$

where  $m$  is in grams, at a radius of 6,374 km. This value was derived from a revised calibration of Giotto dust impact detection system measurements taken 7,544 km from the nucleus<sup>14</sup>. We scaled the 7,544 km law to 6,374 km by assuming a  $1/r^2$  particle number density law in the coma. This  $N(m)$  is about a factor of three higher than that based on an earlier calibration<sup>15</sup>. The slope of the curve is accurate only for grains in the range  $10^{-9}$ – $10^{-6}$  g, but the effect of smaller grains is insignificant in this calculation, and we will assume that this  $N(m)$  does describe larger grains which were poorly sampled by the spacecraft. The emission per mass, assuming the grains to be solid spheres, is

$$E_m = 4\pi a^2 \pi B_v(T) Q(a, T) m^{-1} \\ = 3\pi B_v(T) Q(a, T) (\rho a)^{-1}$$

For silicate grains we take  $\rho = 2 \text{ g cm}^{-3}$ , and approximate  $Q(a, T)$  by<sup>11</sup>:

$$Q(a, T) = 0.56a(1 - \exp(-1.2/a))$$

where the grain radius  $a$  is in microns. Although this absorption coefficient is a Planck mean over all frequencies, it is a good approximation to an integration over our  $10.3 \mu\text{m}$  bandpass because the silicate cross section and the 360 K black body both peak at about this wavelength<sup>16</sup>.

For the upper mass limit we use  $M = 3.3 \times 10^{-3} \text{ g}$ , a value derived from a theoretical model<sup>15</sup>. The resulting volume luminosity is  $L_v = 2.2 \times 10^{-22} \text{ erg cm}^{-3} \text{ s}^{-1} \text{ Hz}^{-1}$ . Integrating along a line of sight passing 6,374 km from the nucleus with an assumed  $1/r^2$  law in the coma gives a predicted surface brightness of  $\sim 80 \text{ mJy arc s}^{-2}$ .

From our photometry, the contrast of the silicate feature above the continuum is about 0.5 mag., or a factor of 1.7. We observe a mean surface brightness of  $\sim 1,300 \text{ mJy arc s}^{-2}$  at a distance of 6,374 km, out of which  $\sim 500 \text{ mJy arc s}^{-2}$  may be attributed to optically thin silicate emission. This is six times the predicted value.

An intriguing explanation for the large discrepancy may be that the grains are not solid spheres, but have a fluffy structure, such as seen in interplanetary dust particles<sup>17</sup> and suggested for cometary grains by Greenberg<sup>18</sup>. Solid silicate grains also could be part of an icy clathrate mixture which releases small grains when heated. Both types of particles would have higher surface area to mass ratios which would raise the predicted luminosity. The clathrate model would also help to explain the disappearance of the silicate feature in several comets at heliocentric distances greater than 1 AU (E. P. Ney and R. D. Gehrz, in preparation).

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## Free precession of the comet Halley nucleus

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Images<sup>1–3</sup> of the comet Halley nucleus by the Vega and Giotto spacecraft and a compilation<sup>4</sup> of recurring events show a 'rotation' period of roughly 2.2 days. In contrast, molecular emission intensities<sup>5</sup> ( $\text{C}_2$  and CN) in the inner coma exhibit 7.4-day periodic variations. In this report the comet Halley nucleus is modelled dynamically as a rigid homogeneous ellipsoid with three unequal principal axes in torque-free rotation about its centre of mass. Two qualitatively-distinct, free-precessional modes are found, each exhibiting both 2.2-day and 7.4-day periodicities. As viewed from the nucleus, the two modes are distinguished by the closed path (called a polhode) that the end of the angular velocity vector traces with a period of 7.4 days (see Fig. 1). The long-axis mode has a polhode that encloses only the long principal axis of the nucleus. It reduces to a motion proposed by Sekanina<sup>6,7</sup> when the minor axes are equal. However, because the polhode encloses the long axis in a long-axis mode, the resulting motion with a 7.4-day period around that axis conflicts<sup>8</sup> with the Vega and Giotto images of the nucleus. The short-axis mode occurs when the minor axes are unequal and the polhode encloses only the short principal axis of the nucleus. The resulting motion is around the short axis and does not conflict with the spacecraft images.

A potato-shaped<sup>1</sup> rigid body with unequal moments of inertia is known to freely precess<sup>9,10</sup> if it is spun about an axis that is oblique to its principal axes of inertia (see Fig. 1). We model the comet Halley nucleus by a dynamically-equivalent, torque-free, rigid, homogeneous ellipsoid with principal axes of unequal

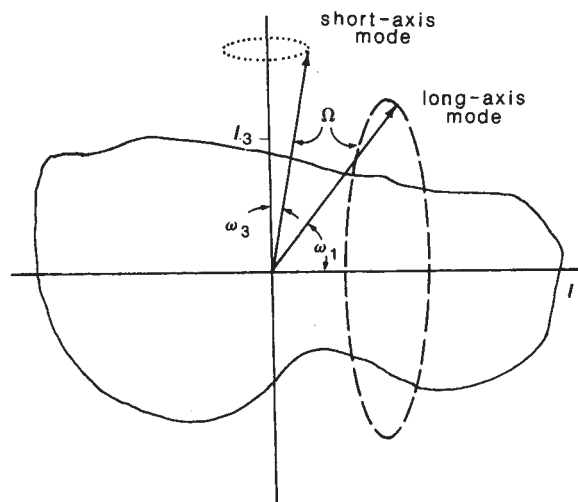


Fig. 1 The profile<sup>8</sup> of the comet Halley nucleus is shown together with the polhodes for the long-axis mode (dashed) and the short-axis mode (dotted).

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lengths  $l_1 > l_2 > l_3$ . We shall follow the notation in ref. 9, and denote by  $\Omega_i$  the component of the angular velocity  $\Omega$  along the moving  $i$ th principal axis of the ellipsoid. If  $\Omega$  is oblique to these principal axes, then the terminus of the vector  $\Omega$  traces a polhode repeating its orientation relative to the nucleus with a period  $T$ , which we shall now compute. The Euler equations<sup>9</sup> determining  $\Omega$  are

$$\dot{\Omega}_1 = -\alpha \Omega_2 \Omega_3$$

$$\dot{\Omega}_2 = \beta \Omega_1 \Omega_3$$

$$\dot{\Omega}_3 = -\gamma \Omega_1 \Omega_2$$

where the dot represents a time derivative and the ratios,

$$\alpha = (l_2^2 - l_3^2)/(l_2^2 + l_3^2)$$

$$\beta = (l_1^2 - l_3^2)/(l_1^2 + l_3^2)$$

$$\gamma = (l_1^2 - l_2^2)/(l_1^2 + l_2^2)$$

involve moments of inertia. The solution for  $\Omega_2$  can be determined by using the two unnamed constants of the motion  $\gamma \Omega_2^2 + \beta \Omega_3^2$  and  $\alpha \Omega_2^2 + \beta \Omega_1^2$  to eliminate  $\Omega_1$  and  $\Omega_3$  from the second Euler equation. The long-axis and short-axis modes are distinguished by the constant (of the motion),

$$m = \frac{\alpha(\gamma \Omega_2^2 + \beta \Omega_3^2)}{\gamma(\alpha \Omega_2^2 + \beta \Omega_1^2)}$$

being less than or greater than unity, respectively. The periods of these modes are, respectively (see equation (37.12) in ref. 9),

$$T = \frac{4K(m)}{\sqrt{(\beta\gamma)\sqrt{[\Omega_1^2 + (\alpha/\beta)\Omega_2^2]}}}$$

and

$$T = \frac{4K(m^{-1})}{\sqrt{(\alpha\beta)\sqrt{[\Omega_3^2 + (\gamma/\beta)\Omega_2^2]}}}$$

where

$$K(m) = \int_0^{\pi/2} (1 - m \sin^2 u)^{-1/2} du$$

is a complete elliptic integral of the first kind.

As the end of the vector  $\Omega$  traces a polhode enclosing either the long or the short axis, we may select a convenient time such that  $\Omega_2$  vanishes and denote  $\omega_i = \cos^{-1}(\Omega_i/|\Omega|)$  evaluated at that time. Thus, we arrive at the formulae for the respective periods,

$$T = \frac{1}{\sqrt{(\beta\gamma)}} \frac{2}{\pi} K\left(\frac{\alpha}{\gamma} \tan^2 \omega_1\right) \sec(\omega_1) \frac{2\pi}{|\Omega|}$$

and

$$T = \frac{1}{\sqrt{(\alpha\beta)}} \frac{2}{\pi} K\left(\frac{\gamma}{\alpha} \tan^2 \omega_3\right) \sec(\omega_3) \frac{2\pi}{|\Omega|}$$

The two modes are also delineated by the argument of the elliptic integral  $K$  being less than unity. For a given total angular momentum, the rotational energy of the nucleus in each long-axis mode is greater than in each short-axis mode.

For equal minor axes the short-axis mode disappears and the long-axis mode reduces to a motion that has been considered by Sekanina<sup>6,7</sup>. In that motion,  $\Omega$  has a constant magnitude and moves at a constant angle  $\omega_1$  to the long axis, encircling it with a 'spin' period

$$T = \frac{l_1^2 + l_3^2}{l_1^2 - l_3^2} \sec(\omega_1) \frac{2\pi}{|\Omega|}$$

The angle  $\theta_1$  between the long axis and the spatially-fixed angular momentum  $M$  is also constant, with

$$\tan(\theta_1) = \frac{l_1^2 + l_3^2}{2l_3^2} \tan(\omega_1)$$

The long axis, the angular velocity  $\Omega$ , and the angular momentum  $M$  remain coplanar at all times, with that plane precessing once around  $M$  with a period

$$T_p = \frac{l_1^2 - l_3^2}{2l_3^2} \cos(\theta_1) T$$

Sekanina<sup>6,7</sup> has identified  $T_p$  with the 2.2-day period deduced from the spacecraft images<sup>1,2</sup> and recurring events<sup>4</sup>. He also identified  $T$  with the 7.4-day period of the emission intensity variations<sup>5</sup> in the C<sub>2</sub> and CN. Moreover, we identify  $2\pi/|\Omega|$  with the period of 2.08 days (50 h) deduced<sup>3</sup> from the shapes of the jets emitted by a rotating nucleus.

Spacecraft images<sup>8</sup> suggest that the nucleus measures roughly  $16 \times 8 \times 7.5$  km with a shape that departs markedly from an ellipsoid. Indeed, the nucleus has<sup>8</sup> "a one kilometre deep depression near the centre of its southern face...". To approximate the nucleus by a dynamically-equivalent ellipsoid, we shall put  $l_1 : l_2 : l_3 \approx 16 : 8 : 7$ . Thus, computing  $\alpha$ ,  $\beta$ , and  $\gamma$ , we arrive at

$$T \approx 1.57 \frac{2}{\pi} K(0.221 \tan^2 \omega_1) \sec(\omega_1) \frac{2\pi}{|\Omega|}$$

and

$$T \approx 3.33 \frac{2}{\pi} K(4.52 \tan^2 \omega_3) \sec(\omega_3) \frac{2\pi}{|\Omega|}$$

in the long-axis and short-axis modes, respectively.

For unequal minor axes, the long-axis mode is qualitatively the same as in the case of equal minor axes<sup>6,7</sup>. In the latter motion, the nucleus spins<sup>6</sup> about its long axis with a period of 7.4 days, while this axis precesses about  $M$  with a period of 2.2 days at a precession angle  $\theta_1 \approx 77^\circ$ . However, Smith *et al.*<sup>8</sup> find that the predicted changes in the profile of the nucleus in each long-axis mode conflict with Vega and Giotto images. That conflict stems from a particular shape that makes<sup>8</sup> the spatial orientation of the nucleus easily recognizable in Vega and Giotto images of adequate resolution. Observing that the profiles obtained 4.7 days apart, can allow only an integer number of rotations about the long axis over an interval of 4.7 days, the authors reject<sup>8</sup> a 7.4-day rotation period about the long axis.

In contrast, the profile of the nucleus in each short-axis mode is not predicted to change markedly, as the relative motion of the angular velocity  $\Omega$  follows a polhode around the short axis, with that axis passing through the depression on the southern face. Thus, there is no conflict with the spacecraft images. Moreover, any 'spin' period  $T$  exceeding  $3.33 \times 2\pi/|\Omega|$  is possible for some  $\omega_3$ , because the range of  $(2/\pi)K$  is  $[1, \infty)$ . In particular, for  $2\pi/|\Omega| \approx 2.08$  days as deduced<sup>3</sup> from the shapes of the jets emitted by a rotating nucleus, and  $T \approx 7.4$  days from the Millis data<sup>5</sup>, we have  $\omega_3 \approx 11^\circ$ .

The short-axis mode, occurring only for unequal minor axes, is an alternative explanation of the conflict between the 2.2-day and 7.4-day periods<sup>1-5</sup> for the comet Halley nucleus. It requires less energy, for fixed total angular momentum, than the long-axis mode being proposed by Sekanina<sup>6,7</sup> and is not immediately rejected by the Vega and Giotto images<sup>8</sup>.

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# Balloon-borne observations of the development and vertical structure of the Antarctic ozone hole in 1986

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A springtime deficit in Antarctic stratospheric ozone has been developing over recent years<sup>1,2</sup>. Here we describe the vertical distribution of ozone which was measured at McMurdo Station, Antarctica (78°S), using balloon borne sensors, on 33 occasions during the period 25 August–6 November 1986. These observations suggest a highly structured cavity confined to the 12–20 km altitude region. In the 17–19 km altitude range, the ozone volume mixing ratio declined from about 2 p.p.m. at the end of August to about 0.5 p.p.m. by mid-October. The average decay in this region can be described as exponential with a half life of about 25 days. While total ozone, as obtained from profile integration, declined only about 35%, the integrated ozone between 14 and 18 km declined more than 70%. Vertical ozone profiles in the vortex revealed unusual structure with major features from 1 to 5 km thick which had suffered ozone depletions as great as 90%.

Satellite data indicate that McMurdo was inside some portion of the vortex throughout the measurement period and was near the temperature minimum on three occasions. Preliminary in-field comparisons with standard ECC ozonesondes launched at South Pole Station, indicated general agreement during the period in which data were compared (19 August–19 September). On several occasions, the aerosol distribution was also measured. These data are currently being analysed and only brief references to preliminary results, where they appear important for interpretation of the ozone data, can be given here.

For the ozone measurements, electrochemical cells and pumps of commercial (Science Pump Corp.) ozonesondes were used. The sonde electronics were replaced with operational amplifiers and voltage-to-frequency converters to produce a digital response. A small bead thermistor was attached to the pump to determine its temperature (required for determining flow rate). The ozone and pump temperature frequencies were read into counters, the output of which were added to the serial data stream of a commercial (Vaisala Corp.) digital radiosonde which provided pressure, external temperature and humidity. A Motorola 6805 microprocessor controlled the data collection and transmission via 403 MHz telemetry. A measurement of all parameters was made at approximately 5 s intervals which provided an ozone integration of about 15 m in the vertical direction during balloon ascent. The average, e-folding response time of the electrochemical sensors is about 20 s so that sub-structures of a few hundred metres in vertical extent could be resolved. The incoming signal was decoded, recorded on floppy disk and analysed in flight using IBM PC compatible hardware. A second, remote telemetry station (free from the surrounding hills at McMurdo which limit usable range in the prevailing easterly direction) was maintained on the annual ice.

The complete ozone payload, including a 30-m reel-down device, weighed 1.8 kg. Linear, low-density, polyethylene balloons of volume 538 m<sup>3</sup>, were used to obtain altitudes in the 30–32 km range. A number of test flights were conducted at Laramie, Wyoming, before Antarctic deployment. These included flights of dual ozone instruments which gave excellent agreement. It is estimated that the flight-to-flight comparative errors are less than 5%. The absolute accuracy depends on altitude, being worse at high altitude due to greater uncertainty in the flow-rate of the air sample and due possibly to other reasons associated with the electrochemical technique; however, in the region of the ozone deficit (12–20 km) the error is believed to be no more than 5%.

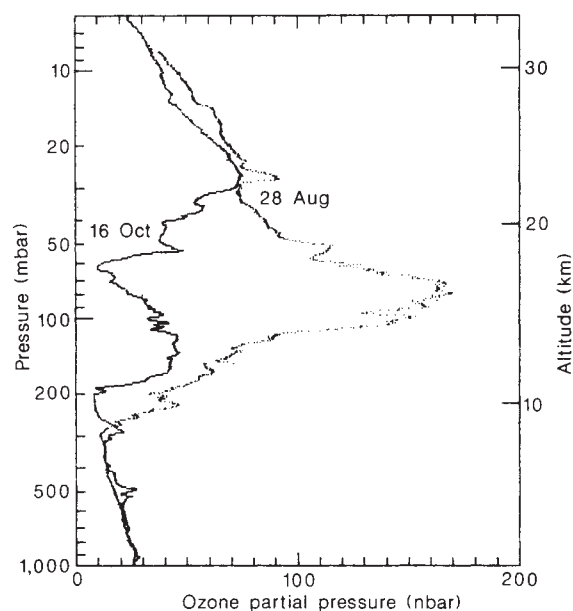


Fig. 1 Ozone partial pressure profiles (McMurdo, 1986) at the start of ozone hole formation and at the height of the depression. Temperatures in the 14–18 km region were  $-80^{\circ}\text{C}$  or lower in both cases, signifying McMurdo's position as being within the region of maximum ozone depletion at these times.

Figure 1 is a comparison of ozone partial pressure profiles in the vortex before major depletion had begun and near the height of the depression. It is possible that some depletion is already present in the August profile at the 20 km level. Total ozone, obtained by integrating these profiles, was 271 and 155 matm cm including an extrapolation at constant mixing ratio above the highest point reached, of 28 and 13 matm cm, respectively. It is estimated that these values are accurate to  $\pm 5\%$ . A Dobson spectrophotometer was not available at McMurdo for comparison. The National Oceanic and Atmospheric Administration (NOAA) Aeronomy Laboratory's visible spectrometer, operated at McMurdo during the period, mainly for  $\text{NO}_2$  measurements but capable of total ozone measurements, indicated these to be reasonable bounds on total ozone for the period. The minimum ozone partial pressure of about 10 nbar on 16 October, at a pressure of about 60 mbar, was the lowest recorded in the stratosphere and represents a depletion in excess of 90% at this altitude. A sounding 3 hours earlier gave nearly identical results. Satellite measurements of total ozone (A. Krueger, personal communication) indicate that the ozone hole was over McMurdo on this day.

The reduction of partial pressure with altitude between 100 and 60 mbar on 16 October in Fig. 1 is faster than would occur if vertical mixing (at constant mixing ratio) were taking place. Thus, it is unlikely that upward motion resulted in this feature. In fact, none of the ozone profiles indicated any mixing of tropospheric air into the stratosphere. Aerosol measurements conducted during the period of ozone depletion showed no evidence of upward motion in the 50–100 mbar region. Quasi-horizontal advection of ozone-poor, tropospheric air from lower latitudes might account for low ozone levels below about 15 km (100 mbar pressure level) but the implication of such a process at 18 km (60 mbar pressure level) is quite doubtful. In addition, aerosol measurements on this day showed no evidence of corresponding layers as would probably be associated with such advection.

Figure 2 shows the time development of the integrated ozone in 2 km intervals. These data show clearly that the depletion was limited to the 12–20 km altitude region, and that it was most severe in the 14–18 km range. Ozone mixing ratio isopleths

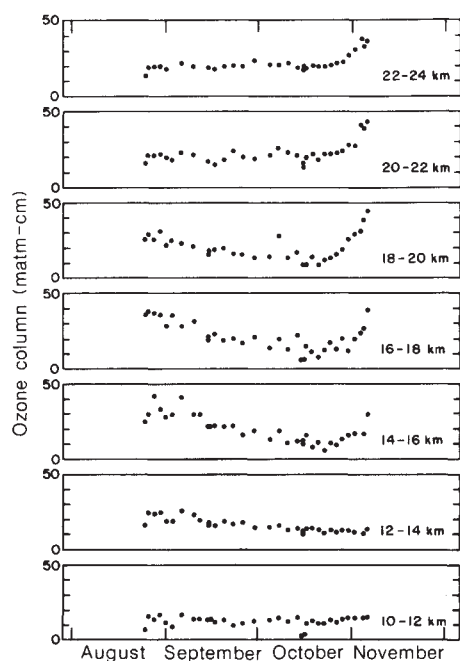


Fig. 2 Two kilometre average columnar ozone against time at McMurdo Station in 1986. Ozone decay is limited to the 12–20 km altitude region.

indicate that the depletion appeared to progress downward in altitude, beginning early and ending early in the 18–20 km region and beginning late and ending late in the 12–14 km region. In the intermediate 14–18 km region, depletion continued through most of the period reaching a deficit in excess of 70% in late October. The increasing ozone trends at high altitude at the end of October are due to appearance of the high ozone ridge over McMurdo at these altitudes, probably associated with the imminent final warming.

Figure 3 gives the time development of the  $18 \pm 1$  km average temperature and ozone mixing ratio, the total ozone column and the 12–20 km ozone column. The temperature data indicate the gradual springtime warming with excessive warming periods when McMurdo was nearer to the boundary of the vortex, notably in early September, early October and finally in late October. Satellite maps of temperature and height at 50, 30 and 10 mbar pressure, obtained within a few days of their production, confirmed the position of McMurdo relative to the temperature minimum and the centre of the low pressure system (the latter two not generally at the same location). While the decline in the total column is not impressive, amounting to only about 35%, the 12–20 km ozone column declined about 68% and the  $18 \pm 1$  km mixing ratio about 75%. The ozonesonde data of Chubachi<sup>3</sup>, obtained at Syowa Station (69° S) in 1982, although including only a few soundings during the critical September period, indicated a decline at 50 mbar (about 18.5 km) from about 3 to 2 p.p.m.v. or about 33%. It appears that the gradient of the decline has increased substantially in the intervening four years. Although the Syowa ozonesonde data had already indicated that most of the depletion occurred near the ozone partial pressure maximum, a sharp upper boundary of the depletion region was not apparent. The vertical ozone profiles of Hofmann *et al.*<sup>4</sup> in the vortex at McMurdo in early November 1985 did not include a reference profile before the depletion began. In the latter case, comparison of vortex profiles with extra-vortex profiles suggested depletion to 30 km. It is now clear that such a comparison is invalid and that one must characterize vortex profiles in August, before the depletion begins.

As indicated in Fig. 3, the mixing ratio decay during late August and September can be represented by an exponential

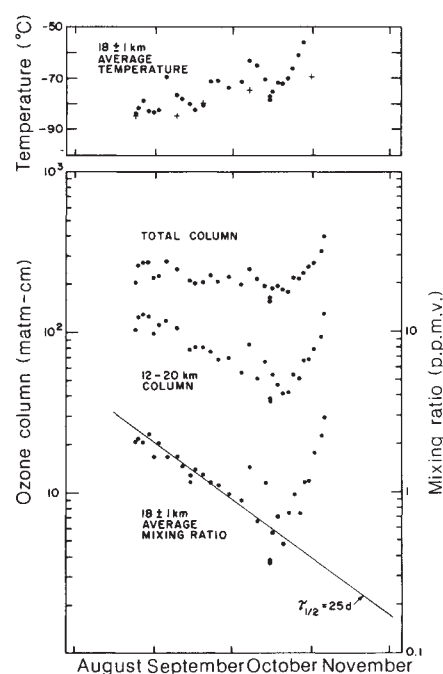


Fig. 3 Total ozone, the 12–20 km columnar ozone, and the temperature and ozone volume mixing ratio averaged between 17 and 19 km against time at McMurdo Station in 1986. The estimated half life for the mixing ratio decay during September is about 25 days. Temperatures indicated by crosses are from satellite data and signify the lowest temperatures observed at 50 mbar in the polar regions.

with a half life of about 25 days (e-folding time of 36 days). It appears that motion of the ozone ridge (region of high total ozone surrounding portions of the vortex) towards McMurdo above 16 km may result in the occasional higher mixing ratios and higher 12–20 km integrated ozone values observed in October. While the available data are insufficient to draw a firm conclusion, it appears that these higher levels may also be decaying to some extent and suggest the importance of measurements in the ridge region.

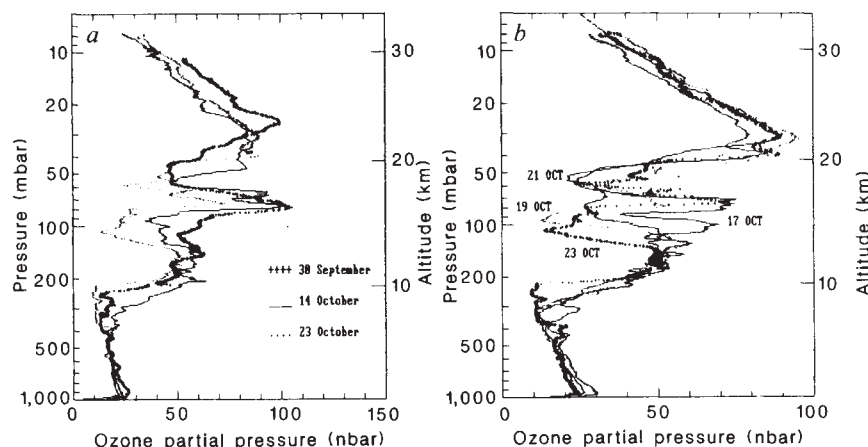
As demonstrated in Fig. 4, the depletion region displayed a great deal of vertical structure especially in the 14–18 km altitude range during October. The depletion was highly heterogeneous with 2–3 km thick layers of high (>75%) ozone depletion interspersed with layers which had suffered only minimal (<25%) losses. While some of these features may be due to advection, it is unlikely that all are because they were not accompanied by correlated temperature changes or by aerosol changes when the latter were measured, which might be expected if they originated outside the vortex.

The minimum ozone region on 16 October (see Figs 1 and 4) seemed to persist for several days suggesting continental proportions although this observation may have been fortuitous since more numerous soundings are necessary to resolve the size of such a feature. It was clearly seen in soundings 3 hours apart. Wind speeds in the depleted region were about  $20 \text{ ms}^{-1}$  at this time which would suggest a horizontal extent of at least 200 km. Figure 4a shows an ozone layer in the heart of the depletion region which re-occurred with a 10–14 day period. Figure 4a also shows the depletion to be most severe at the upper altitudes (18–20 km) early on but to become a dominating feature at the lower altitudes (12–14 km) later on.

Figure 4b is a further example of the high degree of structure in the depletion region during October. Here four soundings, each two days apart, exhibit dramatic variations suggesting small-scale spatial structure. Although these results are from a single station, satellite data indicate that McMurdo was near the minimum temperature (and probably ozone) region in late



**Fig. 4** *a*, Ozone partial pressure profiles which displayed a recurring ozone layer within the hole region at about 16–17 km. Temperature profiles did not show corresponding variations but were uniformly low in this region. *b*, Ozone partial pressure profiles measured 2 days apart showing the high degree of vertical structure present. Profiles such as that of 17 October indicate the presence of numerous sub-regions of depleted ozone with adjacent regions affected to a lesser extent.



August, mid-September and mid-October (see temperature data in Fig. 3). Motion of the minimum ozone region allows sampling of a large portion of the phenomenon at different times. The fact that the decay, as shown in Fig. 3, is quite uniform throughout September, suggests that on a large scale, the phenomenon is quite uniform while on a finer scale, as observed in vertical ozone profiles, considerable structure exists.

The most significant observation in this work is that the majority of the Antarctic ozone depletion occurs between the altitudes of about 12 and 20 km. The upper altitude may be as high as 22 km in the heart of the ozone hole. Subregions appear to exist in which ozone removal is highly efficient, with near total depletion. Integrated over a 2 km region at 18 km, the mixing ratio displays a half life of 25 days. A further important observation is the gradual reduction in altitude of the most efficient depletion region. These observations suggest a relationship with polar stratospheric cloud (PSC) which forms in the stratosphere when temperatures are below about  $-80^{\circ}\text{C}$  (ref. 5). The temporal variation of the ozone decrease at various altitudes in Fig. 2 almost exactly parallels the reduction of PSC extinction against time and altitude given in Hamill *et al.*<sup>6</sup> It appears therefore that PSC evaporation is strongly linked to the ozone depletion phenomenon. Furthermore, the fact that some depletion continues after PSC has disappeared suggests that the evaporation process is involved.

In the cold Antarctic stratosphere, PSC is a frequently occurring optically thin haze which gives extinctions in the  $10^{-3}$  to  $10^{-2}\text{ km}^{-1}$  range. The presence of this haze in the Antarctic stratosphere has been known for some time<sup>7</sup>. On the other hand, nacreous clouds are less frequently occurring distinct and smaller-scale features probably consisting of water-ice crystals. The large extinction which would be derived from optically thick nacreous clouds on relatively rare occasions are generally eliminated in satellite data analysis as the extinction would be considerably greater than  $10^{-2}\text{ km}^{-1}$  (ref. 6).

Owing to the low extinction values associated with PSC observed in satellite data, the uniformity of the extinction when temperatures are less than about  $-80^{\circ}\text{C}$ , and the estimate that a nitric acid trihydrate will condense near this temperature which is about  $5^{\circ}\text{C}$  warmer than for pure water, Toon *et al.*<sup>8</sup> proposed that PSC haze is predominantly composed of nitric acid solution particles (or supercooled droplets). The latter would be smaller than particles derived from pure water due to the relative scarcity of nitric acid. The possible importance of the co-condensation of nitric acid and water was independently proposed by Crutzen and Arnold<sup>9</sup>. In addition to nitric acid condensation, the haze particles will accumulate nitric acid through the heterogeneous reaction of  $\text{N}_2\text{O}_5$ , formed by the reaction of  $\text{NO}_2$  and  $\text{NO}_3$ , with water in the particle during the polar night. Through these processes, most odd nitrogen would be incorporated into PSC haze. Particle sedimentation could thus represent a sink for odd nitrogen<sup>8</sup>.

In addition to being a sink for odd nitrogen, it is possible that inert chlorine reservoir molecules such as  $\text{HCl}$  and  $\text{ClONO}_2$  may also be incorporated in PSC haze and in any nacreous (water) clouds which would form later in the winter in colder regions. Several models<sup>10,11</sup> have been proposed which utilize heterogeneous reactions in PSC to convert these reservoirs to active  $\text{Cl}_x$ . While the models contain no details such as the type of particle involved or the time sequence of events, and probably require that reaction products such as  $\text{HOCl}$  and  $\text{Cl}_2$  be released from the particle before reintroduction of odd nitrogen through PSC evaporation in September, they are able to simulate the magnitude of the depletion. However, the spatial heterogeneity of the depletion, which was mainly observed in October, if not due to advection, can probably not be the result of evaporation of the uniform, satellite-observed PSC haze, nor can it result through chlorine bearing compounds escaping from the particles well before depletion begins. In the latter case, mixing would not allow the observed structure. This suggests the possible importance of more spatially limited nacreous or water cloud and again points to the critical role of particle evaporation.

If molecules such as  $\text{HOCl}$  and  $\text{Cl}_2$  were formed heterogeneously in or on the surface of cloud particles or supercooled droplets<sup>6</sup> but were only released when the latter evaporated, then one can perhaps see how such large depletions in relatively small regions of space might develop since the nacreous clouds would evaporate in early spring before the nitric acid haze evaporated and active chlorine compounds released from the water clouds would have a week or two before odd nitrogen was introduced into the system by later evaporation, at higher temperature, of the nitric acid haze. However, since polar air at  $-80$  to  $-85^{\circ}\text{C}$  may undergo warming by  $5$ – $10^{\circ}\text{C}$  as it circulates in the vortex during September, it is not clear that water clouds should survive very long, possibly undergoing repeated evaporation and re-condensation so that their importance is difficult to evaluate. This may not be the case for nitric acid haze which does not require as low a temperature to exist in the condensed state. Although satellite data suggest the near continuous presence of this haze in the winter Antarctic stratosphere, extinction measurements are made over path-lengths of several hundred kilometres and could mask any structure. Arctic measurements of PSC with airborne lidar have indicated the presence of fine-scale structure superimposed on broader features<sup>6</sup>.

The measurements reported here indicate the importance of obtaining high resolution vertical profile data on both ozone and suitable tracers and have emphasized the possible significance of polar stratospheric cloud in the ozone reduction phenomenon. Modelling of the chemistry which may take place in such clouds is needed. This will require measurements of cloud particle composition and size distribution. In addition, laboratory measurements of relevant heterogeneous reaction rates will be necessary.

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## Long-term climatic changes indicated by crystal growth in polar ice

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The reconstruction of palaeotemperatures from polar ice samples is essentially based on the isotopic composition of the ice. In this paper we propose a new and independent way to obtain such data by using crystal-size-change profiles. From central Antarctica Dome C and Vostok ice cores data we suggest that crystal growth rate is mainly driven by a built-in 'memory' of the surface temperature conditions at the time of deposition. A semi-empirical model of crystal grain growth is proposed, leading to Last Glacial Maximum–Holocene temperature change estimates in good agreement with isotope interpretations. However, the possible palaeoclimatic application of this model suffers some limitations connected in particular with *in situ* strain conditions.

In several ice cores, the Holocene–Last Glacial Maximum (LGM) climatic change, as depicted by isotope profiles, is characterized by a marked decrease in crystal size<sup>1–6</sup>. This effect has been attributed to various factors such as the *in situ* temperature history, strain conditions and the large concentration of impurities in glacial ice<sup>1–4</sup>. We have attempted to explain the correlation between crystal size and climate by examining the crystal-size profile along the Dome C ice core (Fig. 1b). The crystal growth rate ( $K$ ), expressed as  $\Delta(L^2 - L_0^2)/\Delta t$  where  $L_0^2$  is the extrapolated crystal size at time  $t=0$  was obtained by using the chronology given by Lorius *et al.*<sup>7</sup>. The profile (Fig. 1d) shows the same features as the isotope profile (Fig. 1c). A growth rate of  $3.32 \times 10^{-4} \text{ mm}^2 \text{ yr}^{-1}$  was obtained from the regression line  $L^2$  against time in the Holocene stage ( $r=0.92$  for 30 values). Assuming a constant value of  $L_0^2$  over the core, a factor of 3 in  $K$  (or 1.08 in  $\ln K$ ) is observed between the Holocene and LGM periods.

Figure 2 shows the variation of the growth rate with the site temperature in polar firn. To the data from Gow<sup>8,9</sup>, we have added other values for Dome C, Vostok and Dye 3. For Vostok, we used data from Barkov and Lipenkov<sup>5</sup> and the chronology proposed by Lorius *et al.*<sup>10</sup>. Results of the first 800 m of the Dye

3 core, where strain rates remain small, are from Herron and Langway<sup>11</sup> with the chronology given by Reeh *et al.*<sup>12</sup>. For Holocene firn and ice, the relationship between growth rate and temperature obtained from these data is:

$$\ln K = 20.77 - 6286/T \quad (1)$$

where the slope value corresponds to an activation energy  $E$  equivalent to 0.54 eV, a value very close to 0.52 eV given by Gow<sup>9</sup>.

To evaluate the possible *in situ* temperature effect on the  $\ln K$  profile at Dome C, we used the method described by Duval and Lorius<sup>6</sup>, assuming a 12 °C step at the climatic transition although the estimated variation of the surface temperature from isotope profile was only around 9 °C (refs 7, 10, 13). As shown in Fig. 1e, the resulting calculated growth rate is highly damped with a maximum variation in  $\ln K$  of  $\approx 0.30$  compared with the observed variation of 1.08 (Fig. 1d). Moreover, the step changes observed in crystal size within depth intervals of a few decametres such as around 440 m of depth cannot obviously be explained by differences in growth temperature.

The effect of deformation on crystal growth rate is very small in the Dome C core. Indeed, the horizontal shear and vertical strain rates are lower than  $10^{-5} \text{ yr}^{-1}$  and the expected deformation mechanism is probably dislocation creep accommodated by grain boundary migration<sup>14</sup>. Strain energy is expected to be small compared with the driving force for grain growth. Furthermore, the typically equant texture and the absence of undulose extinction observed throughout the 905 m ice core indicates that grain growth is not modified by deformation<sup>15</sup>.

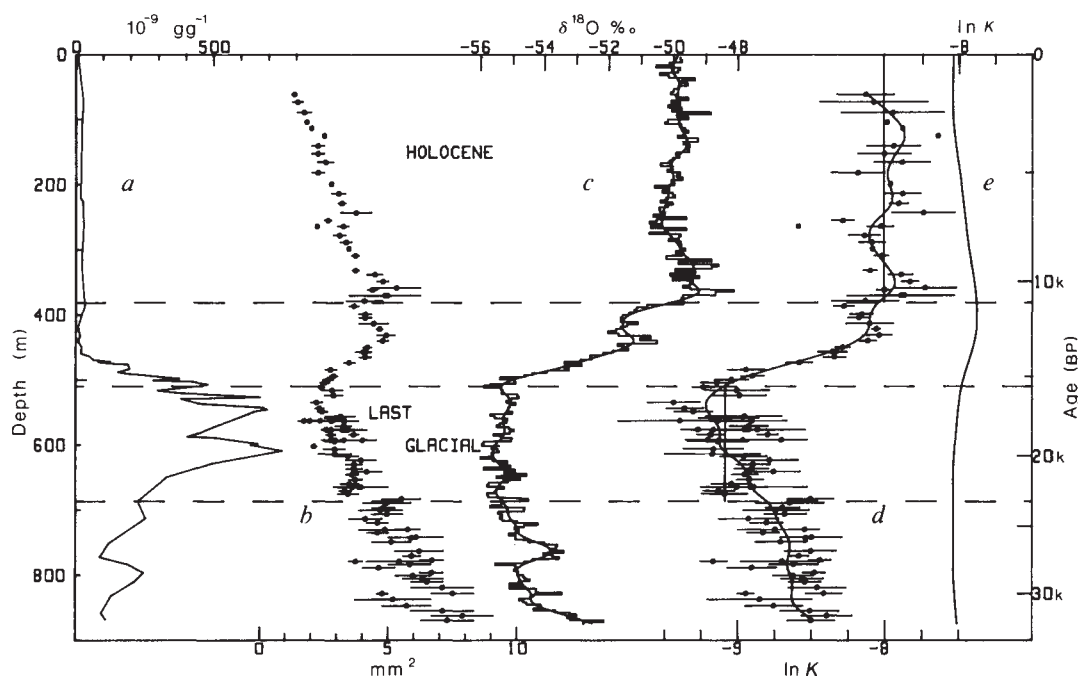
Microparticle concentrations cannot significantly affect overall grain growth in the Dome C ice core although such an effect was observed for very thin layers in some ice cores<sup>1,3</sup>. The drag force induced by particles represents only a small part of the driving force for grain growth at Dome C<sup>6</sup>. In fact, we found that the correlation between crystal size (Fig. 1b) and microparticles (Fig. 1a) vanishes during the last deglaciation between 440 and 400 m and also below 700 m.

The Holocene–Last Glacial Maximum (LGM) transition is also marked by a concomitant change in the concentration of several soluble impurities<sup>16–19</sup> such as sodium, and higher concentrations being associated with colder climatic conditions. But growth rate data in Fig. 2 were obtained from inland and coastal stations for which the concentration of soluble impurities in snow can vary by an order of magnitude. For instance Na values range from about 200 to 400 p.p.b. ( $10^{-9} \text{ g g}^{-1}$ ) near Antarctic coast in Terre Adelie<sup>20</sup> to only 20 to 40 p.p.b. at Byrd<sup>18</sup>, Dome C<sup>17</sup> and Vostok<sup>19</sup> stations. Similar concentrations (20 to 40 p.p.b.) are obtained for Dye 3<sup>21</sup> and Camp Century<sup>16</sup>. If we compare stations with similar temperature but situated differently with respect to the ocean (first in coastal area such as at Maudheim or Wilkes and second inland such as at Dye 3, Camp Century and Byrd), the respective growth rates are similar although sodium contents change by a factor of 5 up to 20. As the observed Na concentration changes along the Dome C ice core are of an order of 5 (refs 17, 22), there is therefore good evidence that sodium cannot significantly affect grain growth. With regard to other major ions, large variations in the concentration of sulphates and nitrates are seen throughout the profile of Dome C core but not especially in the LGM period<sup>22,23</sup>.

Thus the variations in the crystal growth rate along the Dome C core do not appear to result from variations in the *in situ* temperature, strain conditions and impurity content. The strong correlation ( $r=0.89$  for 120 values) we obtained between the crystal growth rate and the isotope content, including ice deposited during glacial time, and the fact that the isotope content is closely related to surface temperature<sup>24,25</sup> suggests that ice particles have a built-in 'memory' of the temperature at the time of deposition at the surface. From isotope data, a LGM temperature of  $-63 \pm 1 \text{ °C}$  was deduced<sup>7,13</sup>. With such a temperature, equation (1) gives a growth rate of  $1.09 \times 10^{-4} \text{ mm}^2 \text{ yr}^{-1}$ , a value which is very close to the mean observed for LGM



**Fig. 1** Dome C ice core profiles versus depth and age. *a*, Insoluble particle content: from ref. 13; *b*, cross-sectional area of ice crystals: from ref. 6 (dots: mean values; bars: one standard deviation); *c*, isotope content ( $\delta^{18}\text{O}\text{‰}$ ) of ice: from ref. 7; *d*, crystal growth rate (dots: mean value; bars: one standard deviation); *e*, estimated effect of the *in situ* temperature



layers (that is  $1.1 \pm 0.05 \times 10^{-4} \text{ mm}^2 \text{ yr}^{-1}$ ). For Vostok, similarly a LGM temperature of  $-65^\circ \pm 1^\circ \text{C}$  (J. Jouzel, personal communication) gives with equation (1), a growth rate of  $0.81 \times 10^{-4} \text{ mm}^2 \text{ yr}^{-1}$  which also compares well with the observed mean value of  $0.89 \pm 0.13 \times 10^{-4} \text{ mm}^2 \text{ yr}^{-1}$  (ref. 5). In both Dome C and Vostok stations, the growth rate of glacial ice therefore appears to be closely related to the surface temperature during LGM. On Fig. 2, the straight line obtained from equation (1) for firn and Holocene ice, when extrapolated to a temperature of  $-70^\circ \text{C}$ , fits very well the measured LGM growth rates of these two stations.

A 'temperature print' can be taken by ice in the first metres of firn while densification of snow proceeds by the packing of grain simultaneously with evaporation-condensation or diffusion processes<sup>26,27</sup>. The high densification rate in this initial stage depends on several parameters including temperature, windspeed and the temperature gradient in the snow.

To explain the anomalous micromechanical and dielectric properties of polar ice, it was proposed that a high concentration of rotational defects (Bjerrum defects) was formed through an ageing phenomenon occurring in the first metres of firn<sup>28</sup>. This ageing was interpreted in terms of some sort of disintegration of ice crystals to lower their free energy. Such a process appears possible under the metamorphism of snow by surface diffusion or vapour transfer between crystals, and also because small-size crystals ( $< 1 \text{ mm}^2$ ) lead to grains of a high specific area. Beyond a critical density of  $0.55 \text{ g cm}^{-3}$  there is a marked reduction in the rate of densification<sup>29</sup>. The decrease of the specific area of grains associated with other densification mechanisms could stop the crystal disintegration. Then the structure of ice would be 'quenched'.

The grain growth is driven mainly by the curvature of grain boundaries. In such a process, the mobility of boundaries is the structure-sensitive parameter: it is directly related to the structure of boundaries via the boundary diffusion coefficient and to the crystal structure via the lattice diffusion coefficient. So, we suggest that the change in the crystal growth rate in polar ice results from variations in the crystal structure induced during the first stage of densification.

To further explain the relationship between the growth rate and temperature, we make two assumptions:

(i) The defects by which the diffusion of water molecules takes place near grain boundaries are interstitials, a mechanism

recently suggested by Higashi *et al.*<sup>30</sup>. The concentration (*c*) expressed in terms of the mean site temperature  $T_s$  is:  $c \approx \exp(-E_f/RT_s)$ ; where  $E_f$  is an apparent formation energy of interstitials.

(ii) The extrinsic point defects formed in polar ice near the surface can disappear only if recrystallization processes occur. In the Byrd ice core extensive recrystallization is initiated below 1,800 m of depth<sup>1</sup>. If, as suggested by Paterson<sup>31</sup>, recrystallization is initiated only above a critical temperature close to  $-10^\circ \text{C}$ , the extrinsic interstitials would be preserved in Dome C core down to an estimated depth of about 2,600 m.

With the assumptions and with  $K \approx c$ , the growth rate can be written as:

$$K \approx \exp(-E_f/RT_s) \exp(-E_m/RT) \quad (2)$$

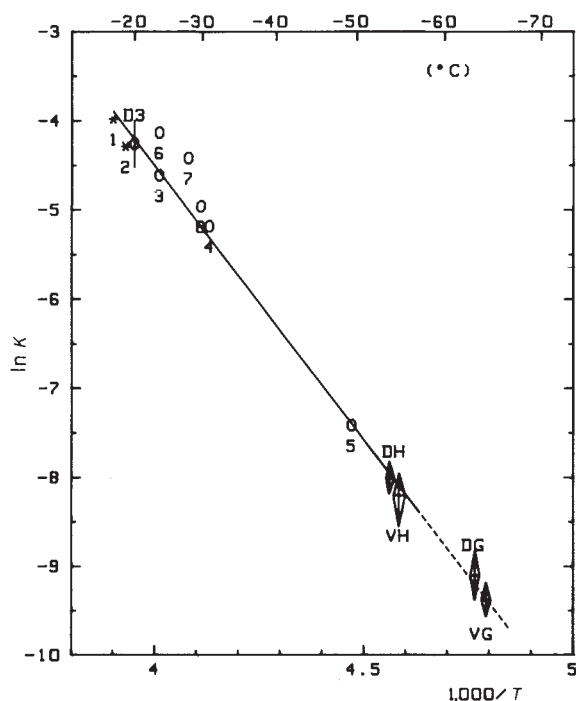
where  $E_m$  is the activation energy for the mobility of grain boundaries and  $T$  the growth temperature. Since in the first metres of firn,  $T$  is equivalent to  $T_s$ , the apparent total activation energy  $E$  in equation (1) can be expressed as  $E = E_m + E_f$ . To obtain the variation of the growth rate with  $T_s$ , an estimation of the apparent activation energy  $E_f$  is needed. From observations of the growth process of dislocation loops in quenched specimens, Higashi *et al.*<sup>30</sup> and Goto *et al.*<sup>32</sup> have deduced the diffusion coefficient of self-interstitials in ice above  $-50^\circ \text{C}$ . They obtained an activation energy for the migration of interstitials of  $0.16 \pm 0.02 \text{ eV}$ . With this value for  $E_m$ , and  $0.54 \text{ eV}$  for  $E_f + E_m$ , the apparent formation energy of interstitials  $E_f$  should be  $0.38 \text{ eV}$ , an estimation which compares well with  $0.40 \text{ eV}$  given by Higashi *et al.*<sup>30</sup>. From equation (2) it is possible to separate the influence of the surface temperature  $T_s$  from that of the growth temperature  $T$ . Taking the logarithm and then the differential of the equation and with  $0.38 \text{ eV}$  for  $E_f$  and  $0.16 \text{ eV}$  for  $E_m$ , we obtain:

$$\Delta \ln K = 0.096 \Delta T_s + 0.040 \Delta T \quad (3)$$

a relationship which should be valid for cold central Antarctica.

The equation (3) based only on growth rate processes can be used as a palaeoclimatic tool. At Dome C the variation of  $\ln K$  between Holocene climate and LGM is about 1.08. The calculated effect of the *in situ* temperature is  $0.10$  ( $\Delta T$  is about  $2.5^\circ \text{C}$ ); so the corresponding value for  $\Delta T_s$  is close to  $10^\circ \text{C}$ .

For Vostok, the variation in  $\ln K$  is 1.17 giving an approximately  $11^\circ \text{C}$  variation in the mean surface temperature. These



**Fig. 2** Ice crystal growth rate against surface temperature in polar ice. Data are from ref. 9: Maudheim (1) and Wilkes (2) are coastal sites (asterisk); all other are from inland areas: site 2 Greenland (3), South ice (4), South Pole (5), Camp Century (6), Byrd (7), Inge Lehman (8). D3 is from the Dye 3 core (mean and one standard deviation); DH and VH are for Dome C and Vostok respectively. These last three values were obtained from ice formed under Holocene climatic conditions. DG and VG are values for Dome C and Vostok ice respectively, from Last Glacial Maximum Conditions, with temperatures obtained from isotope results. The area in each rhombus represents one standard deviation of the mean value.

results are in good agreement with the value of  $\approx 9^\circ\text{C}$  for Dome C and Vostok deduced from an independent approach based on  $\delta^{18}\text{O}$  content<sup>7,10,13</sup>.

The values used for  $E_m$ ,  $L_0^2$  and  $\Delta T_s$  are the main parameters included in the evaluation of  $\Delta T_s$  in equation (3). For  $E_m$  a departure of  $\pm 0.02$  eV from 0.16 eV gives a  $\pm 0.3^\circ\text{C}$  change in  $\Delta T_s$ . LGM  $L_0^2$  values equal to 50% of the Holocene value and LGM layer ages increased by 15% would change  $\Delta T_s$  by  $-1.7^\circ\text{C}$  and  $+1^\circ\text{C}$  respectively. A  $\Delta T$  value  $1^\circ\text{C}$  warmer than the chosen figure would decrease  $\Delta T_s$  by  $0.5^\circ\text{C}$ . In conclusion, the possible variations in these parameters do not significantly change the  $10^\circ\text{C}$  value we obtained for  $\Delta T_s$  at Dome C and Vostok.

As regarding other long-term polar ice records, in the Byrd, Dye 3 and Camp Century profiles, grain growth stops respectively at 450 m (ref. 1), 800 m (ref. 2) and  $\approx 300$  m (ref. 2) respectively. For these cores, strain energy is high enough to prevent grain growth. Extensive undulose extinction observed in the Byrd core<sup>1</sup> supports this assertion. The sharp decrease in crystal size in LGM ice is accompanied by the formation of single pole fabrics in these three cores. This is an indication of ice undergoing rapid shearing. Under such conditions, the surface temperature memory effect on crystal growth is likely to be hidden.

A good check on the empirical model we propose here for the Dome C ice core and validated for the upper part of the Vostok profile will be possible when crystal size data will be available along the 2,083 m profile of this core. Of particular interest will be the crystal growth rates during the last interglacial when the surface temperatures suggested by the isotope data were even higher than the present and those during the penultimate glacial period when temperatures were similar to LGM values<sup>10</sup>.

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## The climate attractor over short timescales

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Recent work has highlighted the possibilities of using certain ideas from the theory of dynamical systems for the study of global climate. These ideas include the geometrical notion of correlation or scaling dimension, first used to analyse attractors arising in mathematical and laboratory systems<sup>1-4</sup> and later applied in a geophysical context<sup>5,6</sup>. The conclusion of the original geophysical work<sup>5</sup> has been criticized in the light of a reanalysis<sup>7</sup> which questions the existence of a low-dimensional climate attractor. Here results of a similar analysis conducted on daily meteorological observations from 1946 to 1982, are announced. This new work overcomes limitations of previous analyses, and supports the existence of such an attractor.

There has been much interest in understanding the properties of chaotic dynamical systems<sup>1,2,4,8</sup>. It has been suggested that strange attractors can be characterized by means of a finite time series sampled from the dynamical system. The correlation function is defined by

$$C(r) = (1/N^2) \sum_{i=1}^N \sum_{j=1}^N \Theta(r - |X_i - X_j|) \quad (1)$$

where  $\Theta(u) = 1$  for  $u > 0$ ,  $\Theta(u) = 0$  for  $u \leq 0$ , and  $\{X_i\}$  is the set of  $N$  sample points on the attractor constructed from a time



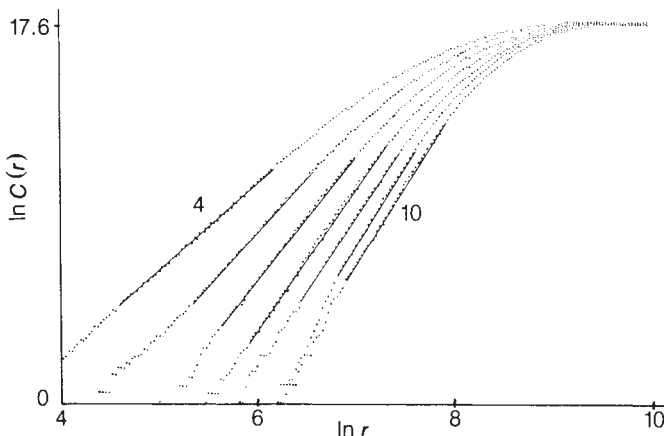


Fig. 1 Plot of  $\ln C(r)$  against  $\ln r$  for embedding dimension,  $D$ , from 4 to 10 as obtained by method 1. Note the convergence of slopes as  $D$  increases.

series of  $M \geq N$  observations. This correlation function counts the number of pairs with distance  $|X_i - X_j|$  smaller than  $r$ . If for sufficiently small  $r$ ,

$$C(r) \sim r^\nu \quad (2)$$

then  $\nu$  is referred to as the correlation dimension of the attractor. If the time series consists only of the sampling of a single dynamical variable from a multivariable dynamical system, the correlation dimension still may be extracted from the series using embeddings consecutively in a sequence of higher dimensional spaces<sup>2,3,4,9</sup>.

This number relates directly to the geometry of the attractor, and has found application in theory and experiment. Moreover, it has been used extensively to characterize the fractal properties of actual physical structures, such as aggregates of particles arising in percolation phenomena<sup>10</sup>.

Experiments on weakly turbulent Couette flow<sup>3</sup> indicate values of  $\nu$  for those systems between 2 and 5 depending on the Reynolds number. Measurements for some turbulent systems reach values as high as 11 (ref. 2). Thus, although a fluid could potentially have a very large number of degrees of freedom, the above studies imply that there are only a few significant degrees of freedom in such complex systems<sup>11</sup>. Despite the fact that it might be difficult to identify these variables, it remains of interest to determine if a system as complex as the atmosphere may be represented by a low dimensional attractor. This dimension number could function as a guide in the development of succinct theories of climate.

Nicolis and Nicolis<sup>5</sup> were the first to address the question of whether a climate attractor exists. They used 500 single variable values of the isotope record of deep-sea cores which cover the span of the past million years. These were interpolated from 184 actual measurements<sup>7</sup>. Grassberger has argued that their low value of  $\nu = 3.1$  is at least in part the result of a smoothing process inherent in completing the data set and does not characterize the actual dynamical behaviour of the climate system<sup>7</sup>. In this work no such filtering is performed. Sets of daily local geopotential values at 500 mb taken at 12 UT extending over a span of 40 years are analysed. The data are over a timescale much smaller than the resolution of the original analysis.

Two variants of the embedding method mentioned above<sup>1,2,4</sup> were executed on nine sets of 12,084 measurements, 108,756 values in all. It was found that for statistical reasons, explained below, a single set corresponding to a single location was insufficient. This is to be contrasted with the 500 values used by Nicolis and Nicolis<sup>5</sup> or the number of values (that is,  $M \leq 7,100$ ) used in the other analyses of this kind<sup>6,7</sup>.

For each embedding dimension,  $D$ , we obtain  $C$  as a function of  $r$ . The region in  $r$  for which

$$C(r) \sim r^p \quad (3)$$

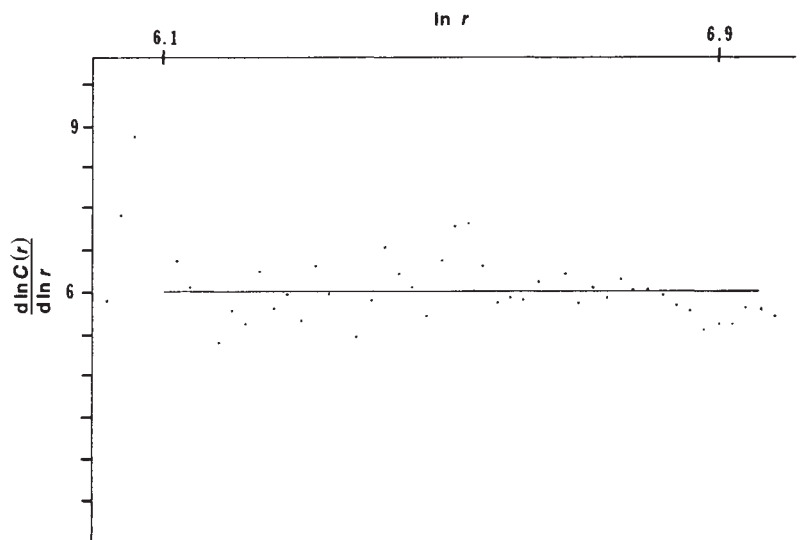
is isolated. This will not cover all  $r$ , as a data set has a finite diameter. As well at the other extreme, the finite resolution and sparseness of the measurements limit the smallest value of  $r$  for which the correlation function can have this behaviour. If  $p$  approaches a limiting value  $\nu$  as  $D$  is increased,  $\nu$  is identified as the correlation dimension.

Care must be taken in carrying out the above programme. Too small a magnitude of  $M$ , may invalidate the procedure. We wish to emphasize that the very existence of this scaling region depends on an adequate value for  $M$ .

In order to understand this, one must begin by recognizing that a particular data set need not exhibit scaling in the form of the asymptotic equation (3) for all or any values of  $D$ . In fact, the only basis for assessing the actual compliance with equation (3) is the quality of a straight line fit through a finite set of points. With an apparently plausible, but erroneous fit of a straight line to a plot of  $\ln C(r)$  versus  $\ln r$ , it is thus possible to obtain spurious values of  $p$ . If a line is fitted to intervals of  $r$  for which  $r$  is too large, the resulting value of  $p$  inferred from such an erroneous fit will be too small, because for large values of  $r$ , approaching the diameter of the finite set  $\{X_i\}$ ,  $C(r)$  saturates, tending towards a constant value. In practice, this situation can be diagnosed by the inability to get a good straight line fit.

Since the number of points within a radius  $r$  of a given point

Fig. 2 A sample plot of  $d \ln C(r) / d \ln r$  against  $\ln r$  showing the scaling region for embedding dimension 9 as determined by method 2.



varies within limits as a positive power of  $r$ , the population of pairs on smaller scales will be much less than those on larger scales. If  $N$ , the number of points, becomes smaller for a fixed  $D$  the population of pairs over the scales for which equation (3) holds will begin to be depleted. Eventually, total depletion on small scales, and large fluctuations in  $C(r)$  because of low populations at slightly larger scales will mask the scaling region entirely. Unfortunately, the remaining segment of  $C(r)$  at still larger values of  $r$  will be somewhat saturated. Any fit to such a curve in this region will result in a value of  $p$  less than its true value, as explained above.

For a fixed  $M$  the number of pairs varies with embedding dimension as  $D^{-2}$ , while the overall 'capacity' of the embedding space increases with  $D$ . Therefore with increasing embedding dimension the relative sparseness of the points will be accentuated on both accounts. The interval in  $r$  for which equation (3) holds is consequently squeezed between the intervals of depopulation and saturation until at some critical value of  $D$ ,  $D_c$ , equation (3) ceases to hold for any  $r$ . Any apparent fit of a straight line to such a curve for  $D > D_c$  will probably result in an underestimate of  $p$ . Thus one might obtain a sequence of underestimates that can lead to a false convergence of  $p$ . For the 500 mb geopotential values, with  $M = 12,084$ , it was found that  $D_c \approx 5$ . That is, the daily values over a forty year interval from one location were insufficient to complete the analysis. In other words, only the values of  $D$  from  $D = 1$  to  $D = 4$  exhibit points on a correct graph of  $p$  against  $D$ . No points for  $D > 4$  exist in this instance, since no good scaling region was evident.

In our analysis we took measures to increase our value of  $D_c$  beyond the values of  $D$  that we analysed. We began by making a boundary correction.  $C(r)$  from equation (1) is referred to as a correlation function; however, in this context this name is perhaps misleading: we only perform the outer sum in equation (1) in order to improve the statistics. In principle, any one of the inner sums would reveal the behaviour of  $C(r)$  as in equation (3), if there were enough data.

The intervals over  $r$  for which scaling occurs will vary strongly from one inner sum to another. An expansion point which is close to the boundary of the set can be surrounded by others only for very small values of  $r$ . Such points contribute to the saturation in  $C(r)$  when  $r$  is still very small. These offending points may be judiciously discarded in the outer sum to produce a boundary corrected function  $C(r)$ . This has the effect of increasing the range in  $r$  over which equation (3) holds, and effectively increases  $D_c$ . For our data  $D_c$  appeared to increase by 1 or 2. This was not enough of an increase in  $D_c$  for our case. With too strong a correction the statistics on low scales suffer badly even if larger  $r$  values participate in the scaling. The result then is simply to displace the scaling interval to larger scales.

In addition to the boundary correction we took the more important step of increasing the overall effective value of  $M$ . We incorporated into the calculation the daily data from eight locations surrounding the original data site. All nine sites fall within a geographic region located in eastern North America, and cover only approximately one per cent of the Earth's surface. We incorporated the extra data into the calculation in two distinct ways. In the first method (method 1), points  $X_i$  in the  $D$ -dimensional embedding space were created by choosing  $D$  consecutive (daily) measurements as coordinates. The data from each location were treated together as independent measurements from a single site by concatenating the different time series from each site to create one large time series. The results of this method are indicated in Fig. 1 and summarized by the crosses in Fig. 3. Figure 1 shows that a straight line fit to the  $\ln C(r)$  against  $\ln r$  graphs is good over a reasonable range of  $r$ .

The other method (method 2) entailed the use of the data from each site as a separate coordinate of a point. The embedding was done by introducing the data from progressively more sites as additional coordinates. In this way the number of points

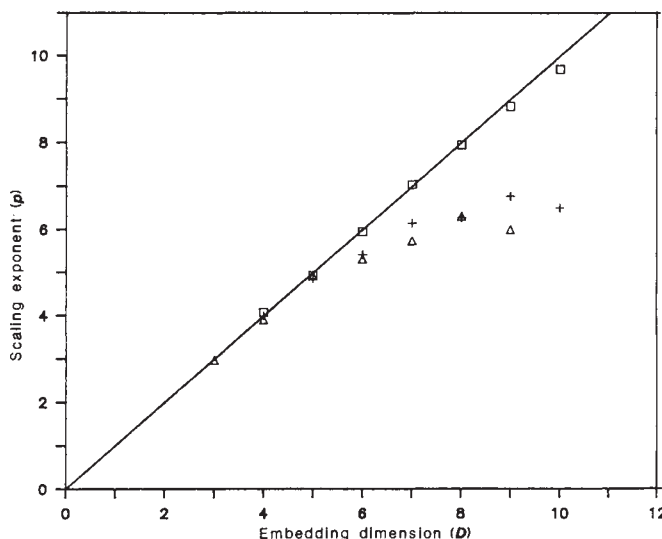


Fig. 3 Summary of results of scaling exponent,  $p$ , as a function of embedding dimension,  $D$ , obtained by two methods (method 1, +; method 2,  $\Delta$ ). A random number set of the same size as the data set was used as a control ( $\square$ ). Note the saturation of the exponent arising from the data, for both methods, while there is no saturation for the random number set. This limiting value is identified as  $\nu$ .

$N$  for each  $D$  remained constant up to  $D = 9$ . The results are indicated in Fig. 3 by the triangles. Figure 2 shows the value of  $p$  at each  $r$  for  $D = 9$  using this method. It is clear that the scaling region, appearing as a plateau marked by the horizontal line in this diagram, is still strongly represented at  $D = 9$ , from which we conclude that  $D_c$  has not been exceeded.

Figure 3 demonstrates that despite the rather different methods employed in selecting points, both methods yield similar results, that is, a similar convergence in  $p$  leading to a value of  $\nu$  slightly in excess of six. The value of  $\nu$  suggests that on short timescales (that is, decades) climate might be represented as a system with as little as seven degrees of freedom. Thus we agree with the original position of Nicolis and Nicolis<sup>5</sup> that the atmosphere and oceans exhibit properties of a low dimensional attractor, although our analysis deals with timescales much shorter than theirs.

Fraedrich<sup>6</sup> has apparently also obtained evidence for a low dimensional climate attractor. However, no convergence of  $p$  was obtained until, perhaps unjustifiably, winter and summer pressure readings were separated. Further, according to our reckoning, the number of measurements used might be considered at best barely adequate for his result.

Since our results are based on measurements fairly localized on the Earth, the question arises as to whether the value of  $\nu \sim 6$  for this attractor is only locally valid. Work on the global nature of the climate attractor is in progress.

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# Effects from equation of state and rheology in dissipative heating in compressible mantle convection

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The thermal profile in the Earth's interior is influenced by many factors. One of the least understood and studied processes is that resulting from adiabatic heating and viscous dissipation. With the exception of the work by Jarvis and McKenzie<sup>1</sup> very little has been done on the effects of compressibility on mantle circulation. It is quite common for geophysicists to add the adiabatic temperature gradient *a posteriori*<sup>2</sup> to temperature profiles derived from Boussinesq (incompressible) equations. Recently it has been shown<sup>3</sup> with the mean-field method<sup>4</sup> that there exists a strong coupling between the rheological parameters, such as the activation volume, and the thermodynamic constants governing adiabatic heating. Here we point out the important consequences brought about by incorporating the effects of equations of state and rheology in the dissipative heating term of the energy equation. We demonstrate explicitly the ways in which compression may raise the interior mantle temperature and illustrate how this effect can, in turn, be used for constraining some of the intrinsic parameters associated with the equation of state in the mantle.

We have employed the mean-field equations for studying steady-state behaviour, which should yield important information about the basic physics of the processes involved. Although it is an approximation to the full set of convective equations, the mean-field approach has increasingly been used by geophysicists to obtain preliminary ideas about various convection problems, ranging from variable-viscosity<sup>5</sup> to double-diffusive convection<sup>6</sup>.

We have derived the single-mode, mean-field equations appropriate for an elastic, compressible convection<sup>1</sup> with variable viscosity in the limit of infinite Prandtl number. Within the present approximation, viscosity depends on horizontally averaged temperature and pressure. A depth-dependent

equation of state  $\rho(z)$ , which increases exponentially with depth, is used for prescribing the background density profile. We have non-dimensionalized, with respect to the whole mantle, depth  $d$  ( $d = 3,000$  km) and have chosen a  $z$ -axis pointing downward. The relevant steady-state equations may be written as the momentum equation

$$\begin{aligned} \frac{d^4 W}{dz^4} + 2 \frac{d^3 W}{dz^3} \left( \frac{1}{\eta} \frac{d\eta}{dz} - 2 \frac{D}{\gamma} \right) \\ + \frac{d^2 W}{dz^2} \left( \frac{1}{\eta} \frac{d^2 \eta}{dz^2} - 5 \frac{D}{\gamma} \frac{1}{\eta} \frac{d\eta}{dz} + 5 \frac{D^2}{\gamma^2} - 2k^2 \right) \\ + \frac{dW}{dz} \left( 3 \frac{D^2}{\gamma^2} \frac{1}{\eta} \frac{d\eta}{dz} - \frac{D}{\gamma} \frac{1}{\eta} \frac{d^2 \eta}{dz^2} - 2k^2 \frac{1}{\eta} \frac{d\eta}{dz} - 2 \frac{D^3}{\gamma^3} + 4k^2 \frac{D}{\gamma} \right) \\ + k^2 W \left( \frac{1}{\eta} \frac{d^2 \eta}{dz^2} + k^2 + \frac{D}{\gamma} \frac{1}{\eta} \frac{d\eta}{dz} - \frac{2D^2}{3\gamma^2} \right) \\ + k^2 \exp \left( 2 \frac{D}{\gamma} z \right) R \frac{\theta}{\eta} = 0 \end{aligned} \quad (1)$$

The temperature field is decomposed into a horizontally averaged part  $\bar{T}(z)$  and a fluctuating component  $\theta(z) \cos kx$ , where  $k$  is the dimensionless wavenumber of the assumed roll along the  $x$ -direction. The equations for the mean and fluctuating temperatures in which the weak-coupling approximation<sup>4</sup> is assumed for the divergence of  $u\theta - \langle u\theta \rangle$ , take the form

$$\begin{aligned} \frac{1}{\rho(z)} \frac{d^2 \bar{T}}{dz^2} - \frac{d}{dz} (\theta W) + D\theta W + \mu \\ + \frac{D\eta}{R} \exp \left( -3 \frac{D}{\gamma} z \right) \left[ \left( 2 \frac{dW}{dz} + \frac{D}{\gamma} W \right)^2 \right. \\ \left. + k^2 \left( W + \frac{1}{k^2} \frac{D}{\gamma} \frac{dW}{dz} - \frac{1}{k^2} \frac{d^2 W}{dz^2} \right)^2 + \frac{1}{3} \left( \frac{D}{\gamma} W \right)^2 \right] = 0 \end{aligned} \quad (2)$$

and

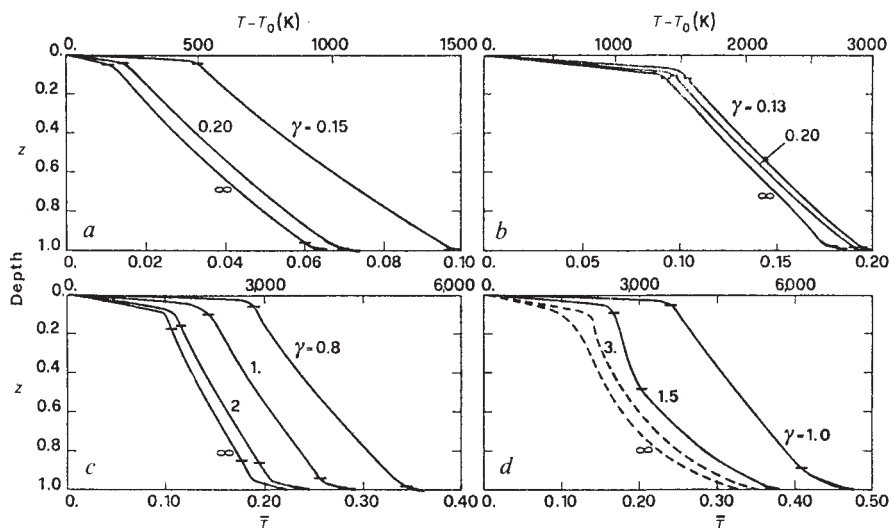
$$\frac{1}{\rho(z)} \left( \frac{d^2 \theta}{dz^2} - k^2 \theta \right) + D(\bar{T} + \bar{T}_0) W - W \frac{d\bar{T}}{dz} = 0 \quad (3)$$

In equations (1)–(3), the mean viscosity profile is given by  $\eta$ . The Rayleigh number  $R$  is based on the interior viscosity  $\eta_\infty$ . The parameters  $D$  and  $\gamma$  are respectively the dissipation number and Gruneisen parameter associated with the equation of state<sup>1</sup> which is

$$\rho(z) = \rho_0 \exp(D/\gamma z), \quad (4)$$

where  $\rho_0$  is the surface density. The scale-height for the adiabatic temperature is given by  $1/D$ , whereas that for the density is

**Fig. 1** Mean temperature as a function of depth for various rheologies. *a*, Constant viscosity,  $\eta = \eta_\infty$ ; *b*, temperature-dependent viscosity,  $\eta(\bar{T})$ ; *c* and *d*, temperature- and pressure-dependent viscosity,  $\eta(\bar{T}, p)$ . A heat flux, based on the conduction flux from volumetric heating with strength  $H$ , is imposed at the bottom. The Rayleigh number for variable viscosity is based on the asymptotic viscosity  $\eta_\infty$ , and is  $10^9$  for all cases. The temperature has been non-dimensionalized by  $\bar{T} = T - T_0/T_D$  where  $T_D = \rho_0 H d^2 / k_0$ . The density  $\rho_0$  is set to  $3,400 \text{ kg m}^{-3}$ ,  $H = 10^{-12} \text{ W kg}^{-1}$  and  $k_0 = 4 \text{ W m}^{-1} \text{ K}^{-1}$ . The surface temperature  $T_0$  is set to  $1,100 \text{ K}$  for all cases to simulate the effects of lithospheric lid from variable viscosity.  $D = 0.5$  and  $\mu = 0$ . The rheological parameters correspond to  $Q^* = 50 \text{ kcal mol}^{-1}$  in *b*, *c* and *d*,  $V^* = 4 \text{ cm}^3 \text{ mol}^{-1}$  in *c* and  $v^* = 8 \text{ cm}^3 \text{ mol}^{-1}$  in *d*. An aspect ratio of one is assumed for the convective roll.



prescribed by  $\gamma/D$ .  $\bar{T}_0$  is based on the surface temperature  $T_0$ , an intrinsic parameter in compressible convection. The parameter  $\mu$  expresses the rate of internal heating. For a situation with a bottom temperature  $T_B$  specified and a given amount of internal heating  $H$ ,  $\mu = R_H/R_B$ , where  $R_H$  represents the Rayleigh number describing a purely internal-heating configuration with an adiabatic bottom<sup>7</sup> and  $R_B$  is the Rayleigh number associated with a base-heated convection with a temperature difference of  $T_B - T_0$ . For a bottom thermal condition with a given flux  $F$  the partitioning ratio  $\mu$  becomes  $\mu = \rho_0 H d / (\rho_0 H d + F)$ .

In the momentum equation both the horizontal gradients of temperature and perturbation pressure are included in the generation of the vorticity. The adiabatic heating term, which is proportional to  $D$  and the vertical thermal advection, appears in both the mean and fluctuating temperature equations.

We use a viscosity law, non-dimensionalized with respect to  $\eta_\infty$ , of the following form

$$\eta(T, z) = \exp\left(\frac{H^*}{RT} - \frac{Q^*}{RT_\infty}\right) \quad (5)$$

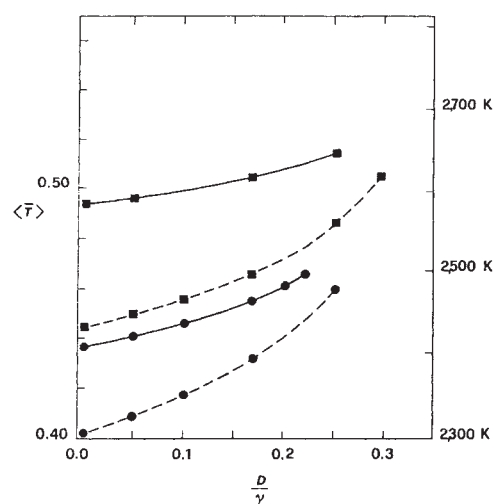
where  $\eta_\infty$  is taken to be  $10^{22}$  P, the activation energy is denoted by  $Q^*$ , the activation enthalpy by  $H^* = Q^* + \rho(z)gzdV^*$ , the activation volume by  $V^*$ ;  $T$  is in Kelvin,  $T_\infty$  is 2,773 K and  $R$  is, in this equation only, the gas constant. The parameter  $T_\infty$  represents a characteristic temperature in the lower mantle. An activation energy of 50 kcal mol<sup>-1</sup> is used for the purpose of illustrating the strong temperature dependence of the viscosity, and activation volumes between 4 and 8 cm<sup>3</sup> mol<sup>-1</sup>, characteristic of deep mantle material<sup>8</sup>, have been employed. An aspect-ratio one cell ( $k = \pi$ ) has been assumed throughout, as have been the stress-free boundary conditions at the top and the bottom. Our results are relatively constant for  $k$  between 1.5 and 9. The temperature at the surface is kept constant at  $T_0 = 1,100$  K, thus imposing  $\theta = 0$  there. At the bottom we have set  $d\theta/dz = 0$  and have employed  $d\bar{T}/dz = 1 - \mu$  for heating configuration with a specified heat flux and  $\bar{T} = 1$  for the fixed bottom temperature situation. We have solved the nonlinear system (1) to (3) with the associated eight boundary conditions on  $\bar{T}$ ,  $w$ ,  $\theta$  by decomposing them into a coupled system consisting of two fourth-order, boundary-value problems. Two hundred grid points have been distributed in an adaptive manner.

The effects of volumetric changes on dissipative heating are manifested by terms involving  $D/\gamma$  in equation (2). From scale analysis this contribution  $\Phi_B$  from the equation of state in the shear-heating term is predicted to behave as

$$\Phi_B \sim \eta_M u_M^2 D^2 / \gamma^2 \exp\left(\frac{D}{\gamma} z\right) \quad (6)$$

where  $\eta_M$  and  $u_M$  denote the characteristic viscosity and velocities respectively in the interior. It is clear that there will be a growing amount of shear heating, as one increases the compressibility or, conversely, decreases  $\gamma$  sufficiently.

Figure 1 shows the mean temperature profiles for various rheologies ranging from one with constant viscosity to a temperature- and pressure-dependent one. A constant heat flux is supplied at the bottom. A moderate value of the dissipation number is used, and the effects of compressibility are brought out by varying  $\gamma$  from  $\infty$  to  $\sim 0.1$ . The extreme cases would correspond to a change in density by a factor  $> 10$ . The tick bars delineate the portions of the thermal profile which lie along the adiabatic gradient. Dashed curves (Fig. 1d) represent super-adiabatic states. Note that in the mean-field approximation viscous dissipation is not entirely balanced by adiabatic heating in the steady equation for the mean temperature. This approximation leads to an increased temperature gradient at the top boundary. The differences in the interior temperature between mean-field and two-dimensional results<sup>1</sup> increase with the amount of convective vigour for constant viscosity<sup>3</sup>.



**Fig. 2** Effects of varying the Grueneisen parameter on the vertically averaged temperature,  $\bar{T}$ . The dissipation number  $D$  is constant at 0.5. The bottom is kept at a constant temperature of 4,100 K;  $T_0$  is the same as in Fig. 1. The temperatures on the right hand side correspond to the averaged temperature given in dimensional form. The nondimensional temperatures on the left are scaled by the temperature drop across the mantle which is here taken to be 3,000 K. The rheological parameters correspond to  $Q^* = 50$  kcal mol<sup>-1</sup> and  $V^* = 4$  cm<sup>3</sup> mol<sup>-1</sup>. The basal-heating Rayleigh number  $R_B$  appears in both the momentum and energy equations, whereas the internal-heating Rayleigh number  $R_H$  appears only in the mean temperature equation. They are both nondimensionalized by  $\eta_\infty$ . An aspect ratio of one is used. ---,  $R_H = 0$ ; —,  $R_H = R_B$ ; ■,  $R_B = 2 \times 10^8$ ; ●,  $R_B = 10^9$ .

The convective strengths of the runs presented in Fig. 1a-c are  $\sim 10^5$  times supercritical. For both constant and temperature-dependent viscosities (Fig. 1a, b) there are no discernible effects from compressibility until  $\gamma$  becomes  $< 0.2$ . As the Grueneisen parameters of mantle substances lie between 1 and 3 (refs 9, 10) these results suggest that shear heating in the presence of compressibility does not result in major changes to the geotherm for constant<sup>1</sup> and temperature-dependent rheologies.

On the other hand, the situation is quite different with the introduction of pressure dependence into the rheology. The shear heating now peaks sharply in the lower mantle. The interior of the mantle can be warmed up by several hundred degrees above the incompressible ( $\gamma = \infty$ ) profile, for reasonable values of  $\gamma$  (Fig. 1c) corresponding to density changes of around 30% across the mantle. For activation volume  $V^* = 4$  cm<sup>3</sup> mol<sup>-1</sup> there is a rapid increase in the interior temperature as  $\gamma$  drops from 2 to 1. For  $\gamma < 1$ , the steady-state solution jumps to a higher branch in which the interior temperature is several thousand degrees hotter. In these cases there is a sharp focusing of viscous heating at the base of the mantle. Convection becomes more sluggish<sup>6</sup> with larger activation volume<sup>3</sup>, leading to a hotter interior (Fig. 1d). However, the transition value of the Grueneisen parameter  $\gamma_c$  still remains within geophysically reasonable limits, between 1 and 2. We remark here that the temperature estimates are based on the cartesian formulation and that effects from sphericity<sup>11,12</sup> would reduce these interior temperatures by around a factor of two in the case of constant viscosity. The transitions at low Grueneisen parameters is also found for Rayleigh numbers 50 and 100 times smaller than the ones reported here.

The combined effects of both basal and partial internal heating configurations are shown in Fig. 2, where the averaged temperature is plotted as a function of  $D/\gamma$  for purely base-heated (dashed curve) and partially internally heated (solid curve) cases. In the latter case the internal-heating Rayleigh number  $R_H$  is set equal to the basal-heating Rayleigh number  $R_B$  ( $\mu = 1$ ). A temperature difference of 3,000 K has been imposed across



the mantle. We observe a gradual increase of the interior temperature with increasing compressibility, as  $D$  is kept constant at 0.5. The temperature rise because of shear heating is greater in the case without any radioactive heating. The last points, shown on the right, mark the regime in which heating effects from volumetric deformations are still small. We observe that, for this set of rheological parameters ( $Q^* = 50 \text{ kcal mol}^{-1}$  and  $V^* = 4 \text{ cm}^3 \text{ mol}^{-1}$ ), the averaged temperature of the mantle cannot exceed 2,700 K, before transition to a much hotter, geophysically unrealistic state would occur for  $D/\gamma$ , greater than about 0.3 or  $\gamma_c = 1.7$ . As this  $\gamma_c$  lies within geophysically plausible bounds, this would argue for the potential importance of employing the equation of state in conjunction with temperature- and pressure-dependent rheology in compressible convection models for constraining the thermodynamic parameters  $D$  and  $\gamma$  of the deep mantle. Conversely, one may also put bounds on the rheological parameters  $Q^*$  and  $V^*$  of the lower mantle, if the equation of state parameters can be estimated with greater precision.

We conclude that the coupling between variable viscosity and equation of state in dissipative heating is potentially an important mechanism in mantle convection. The fundamental concept that rheology, equation of state and radiogenic heating are all linked to each other by virtue of nonlinear thermomechanical couplings must be strongly emphasized as a consequence of these findings. To explore further the implications of this phenomenon for the Earth's thermal behaviour, we need to corroborate these results with the full equations and also with depth-dependent thermal expansion coefficient and thermal conductivity, which would have important implications for the  $D^{11}$  layer. With the next generation of supercomputers, the spatial resolution required to understand this nonlinear process in two or three dimensions is well attainable.

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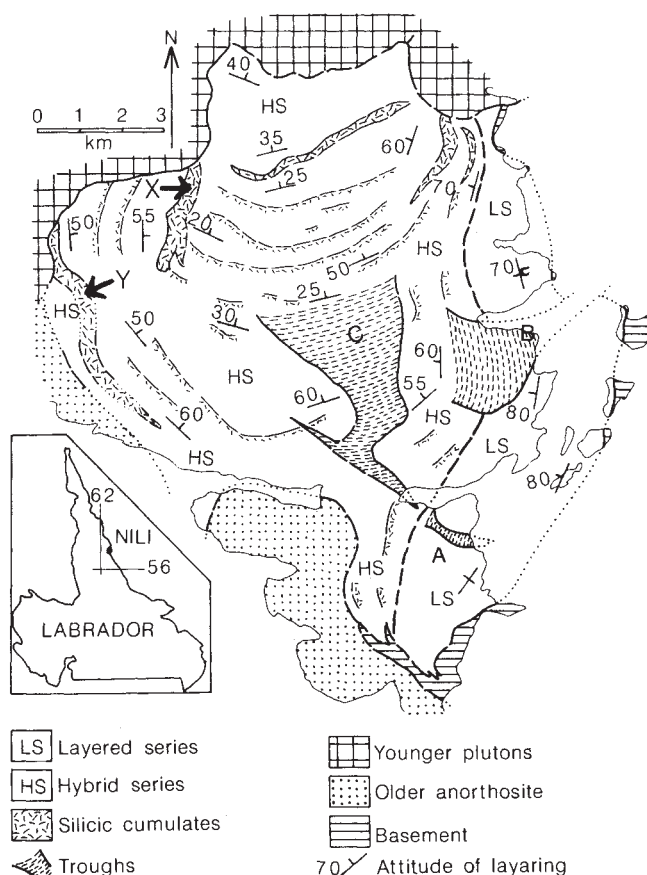
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## Rupture and inflation of a basic magma chamber by silicic liquid

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The prevailing view among volcanic geologists is that the introduction of basic magma into a silicic magma chamber may trigger explosive silicic volcanism<sup>1</sup> and lead to the eruption of mixed magmas<sup>2</sup>. Although the patterns of basic and silicic eruptions in some areas may support an input of basic magma<sup>1</sup> just before silicic eruption, evidence from the study of the erupted rocks is generally equivocal; many occurrences are as readily explained by injection of silicic into basic magma. Although this latter sequence is a more efficient way to produce contemporaneous magma mixing and explosive silicic volcanism, it appears to have been largely overlooked. This report describes evidence in the Newark Island layered intrusion for the rapid emplacement of large volumes of silicic magma into a basic magma chamber and



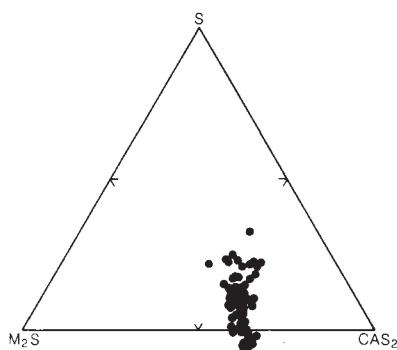
**Fig. 1** Simplified geological map of the Newark Island layered intrusion. Troughs truncate and displace cumulates (both LS and HS) and consist largely of massive gabbroic rocks with walls lined by granite. They originated as silicic feeders, which were then filled by collapsing resident basic magma.

suggests that such events, in the uppermost crust, may also be important in triggering explosive silicic volcanism and commingling of magmas just before eruption.

Layered intrusions, as solidified magma chambers, provide direct evidence for the sequences and compositions of replenishment and mixing events that are sometimes inferred from erupted rocks. Clear proof of injections of basic magma into a silicic chamber can be seen where chilled basic pillows and sheet-like masses are interlayered with silicic cumulates<sup>3,4</sup>. However, when silicic magma is emplaced into a basic magma chamber, it rises rapidly to the top of the chamber and may leave a less obvious record of its passage within the cumulates. If the temperature contrast is great, some resident basic magma will be chilled during the rise of silicic magma and sink to the chamber floor as pillows resting in mafic or hybrid cumulates. Such features are common in the hybrid layered intrusions of the Nain anorthosite complex<sup>5,6</sup>.

The Newark Island layered intrusion (NILI) is ~150 km<sup>2</sup> in area (Fig. 1) and was emplaced at a depth of ~8-10 km (ref. 7). Its original floor, now the eastern contact, rests on Archaean metamorphic rocks; its top is truncated by younger plutons. The Layered Series (LS) consists mainly of gabbroic cumulates, with plagioclase and olivine the dominant cumulus phases<sup>8</sup>. Current-related structures indicate that when these rocks were deposited, the chamber floor dipped moderately to the west. Reversals in mineral composition trends indicate that the magma chamber was periodically replenished by basic magma.

The Hybrid Series (HS) consists mainly of many repetitions of gabbroic cumulates which grade upward through heterogeneous pyroxene diorite and monzonite to rocks with cumulus quartz. During deposition of the HS, the magma chamber



**Fig. 2** Compositions (mol %) of 90 dykes and chilled pillows projected into CMAS (CaO-MgO-Al<sub>2</sub>O<sub>3</sub>-SiO<sub>2</sub>) after Grove *et al.*<sup>15</sup>. M<sub>2</sub>S, olivine; CAS<sub>2</sub>, plagioclase; S, quartz; compositions are projected from CMS<sub>2</sub> (clinopyroxene). Compositions lie along an olivine-plagioclase cotectic, indicating that the liquid compositions were controlled by cotectic crystallization of olivine and plagioclase. These rocks have compositions appropriate for the magmas that produced mafic cumulates in the NILI.

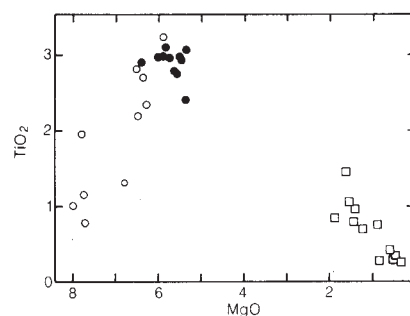
expanded westward and evolved into a north-plunging trough. Layers of silicic cumulates occur at several levels (Fig. 1). Mafic layers resting on them are marked by a strongly chilled and pillowed base that records replenishment of basic magma into the chamber when it was floored by silicic magma. Silicic cumulates on the walls of the chamber (Fig. 1) have sharp outer (basal) boundaries with gabbroic wall cumulates; this abrupt change to silicic cumulates must record the entry of new silicic magma into the chamber and the downward displacement of basic magma. As a result of multiple basic and silicic replenishment, the magma chamber was probably compositionally stratified most of the time.

Basic dykes, ranging in thickness from 1 to 10 m, occur at all levels, and are essentially identical in composition to chilled basic rocks in the HS. Granitic dykes range in thickness from 1 to ~100 m. All appear to be feeders to the NILI.

Three large transgressive bodies ('troughs') occur within the NILI (Fig. 1). Their steep sides sharply cut cumulates; their tops are conformable with overlying cumulates. They contain mainly massive, variably chilled gabbroic rocks, but their margins are lined with fine-grained granite up to several metres thick. The boundary between granite and gabbro is marked by a broad zone (1–20 m thick) of chilled mafic pillows which grades inward to gabbro. These relations indicate that the troughs were initiated as silicic feeders and subsequently became filled with basic magma.

Mafic dykes and chilled pillows have compositions that are appropriate for the liquids that produced the mafic cumulates in the NILI<sup>9</sup>. Their compositional variation suggests dominant control by cotectic crystallization of olivine and plagioclase, the main cumulus minerals in the NILI (Fig. 2). The mafic dykes are more primitive than chilled pillows within the troughs (Fig. 3), and no pillows have primitive compositions. The more primitive chilled liquids (dykes) represent basic magma that replenished the magma chamber; the more fractionated chilled liquids (trough pillows) represent magma that evolved within the chamber. The fine-grained granites in the dikes and troughs approach minimum melt compositions. Their compositions are appropriate for magmas that produced silicic cumulates in the HS.

Sequences of layers along the steep walls of the HS record evidence for a major replenishment of silicic magma. Gabbroic cumulates along the western wall pass abruptly inwards to a thick cumulate layer of two-pyroxene quartz monzonite (X in Fig. 1). Further in, chilled mafic rock occurs in sharp contact with and includes rounded masses of the quartz monzonite. The mafic rock coarsens inward and acquires cumulate textures. A thinner but similar sequence occurs in the eastern wall (Fig. 1).



**Fig. 3** Plot of MgO against TiO<sub>2</sub> for chilled basic pillows from trough C (●), basic dykes (○) and fine-grained granitic rocks (□), including both dykes and the matrix to basic pillows in troughs. All rocks plotted have compositions that closely approximate liquids related to the NILI.

This sequence of layers developed in the following manner. First, a basic magma filled the chamber to produce the outer mafic cumulates. This magma was suddenly displaced downward (because of its higher density) by a replenishment of silicic magma which produced the silicic wall cumulates. A later replenishment of basic magma displaced this silicic magma upward, engulfed some silicic wall cumulates, and produced the inner mafic cumulates.

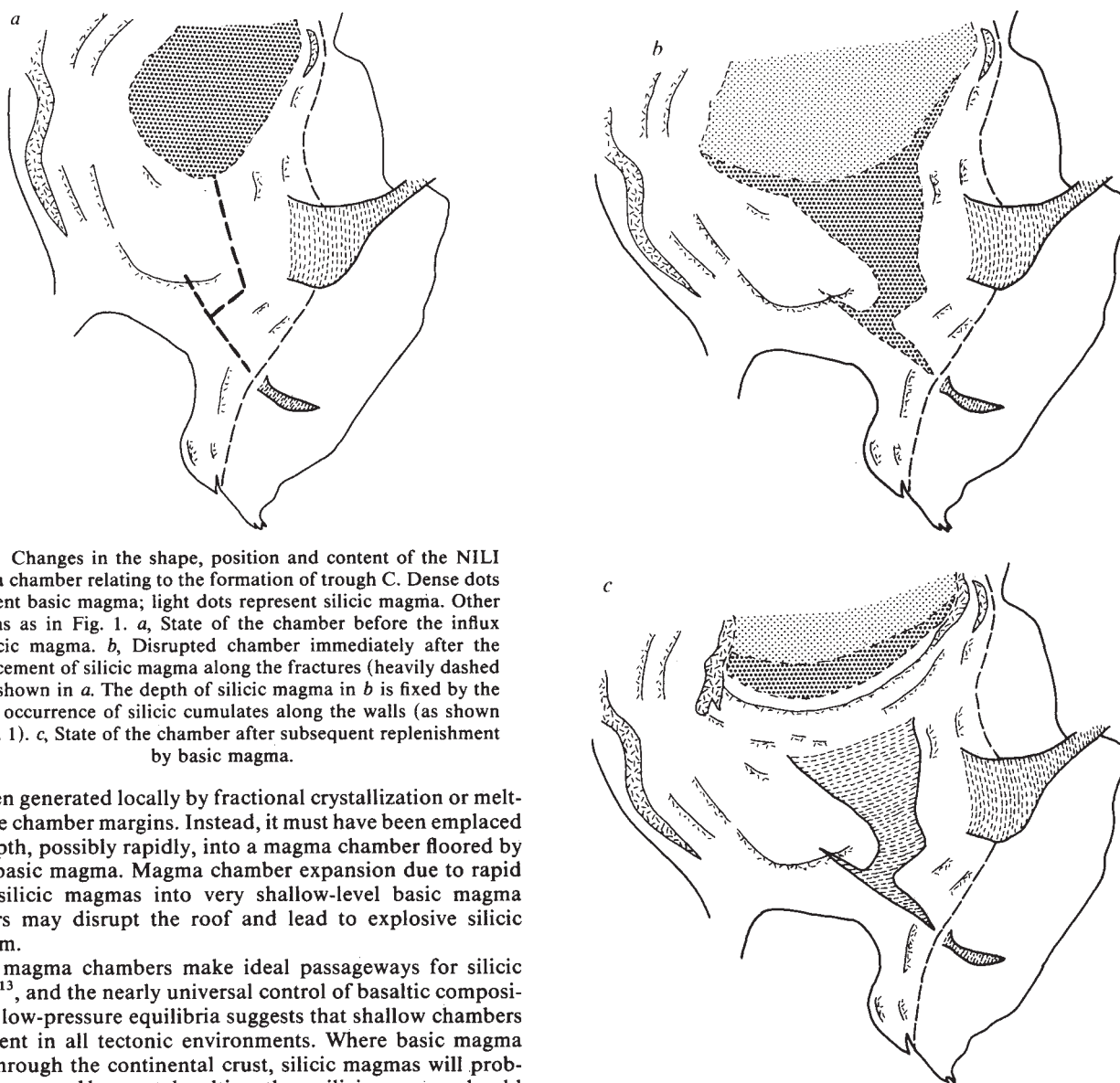
The downward displacement of basic magma by silicic magma can be explained readily if that event corresponds to the silicic influx related to the formation of trough C (Fig. 1) and if, as the compositions of chilled pillows in the trough suggest, the gabbroic rock in the trough crystallized from collapsed resident basic magma. Silicic cumulates at lower level (Y in Fig. 1) can be similarly related to trough B.

The formation and expansion of trough C strongly displaced the existing cumulates and caused a major expansion of the magma chamber (Fig. 1). The abrupt inward transition along the walls from basic to silicic cumulates and the lack of evidence for multiple silicic injections within trough C suggest that silicic replenishment occurred in a single pulse. The size of the trough and the great contrast in densities between resident basic and new silicic magmas suggest that emplacement and collapse occurred rapidly, probably on the scale of hours.

These relations demonstrate that a basic magma chamber was invaded from below by a large volume of silicic magma which was emplaced along fractures in the existing cumulates. Although there are not enough data to specify a unique three-dimensional solution to the disruption of the chamber and, therefore, to the volume of new silicic magma, it is possible to present a reasonable solution in two dimensions based on the presently exposed section of the intrusion. Figure 4 provides three such sections, which trace the evolution of the chamber from before the formation of trough C to after a succeeding replenishment of basic magma. The new silicic magma shown in Fig. 4b covers an area of ~20 km<sup>2</sup>. Assuming a minimum mean thickness of 1 km, at least 20 km<sup>3</sup> of new silicic magma was emplaced at the time of trough formation. This estimate is conservative because, with the roof removed, more silicic magma may have existed at higher levels of the chamber or erupted from the chamber. Figure 4c illustrates the state of the magma chamber after some side-wall crystallization of silicic magma and further replenishment of basic magma.

The rapid upward movement of silicic magma, as recorded in the NILI, offers an attractive alternative to some current interpretations of explosive silicic volcanism and occurrences of magma mixing. Indirect evidence of rapid movement of silicic magma from depth has been noted in some recent studies<sup>10,11,12</sup>. Large volumes of silicic magma may have risen rapidly from depths of 25–30 km to erupt to form the Fish Canyon tuff<sup>10</sup>. At least 27 km<sup>3</sup> of rhyolite magma evolved and segregated in 15,000 yr beneath Santorini<sup>11</sup>. This amount of magma could not





**Fig. 4** Changes in the shape, position and content of the NILI magma chamber relating to the formation of trough C. Dense dots represent basic magma; light dots represent silicic magma. Other patterns as in Fig. 1. *a*, State of the chamber before the influx of silicic magma. *b*, Disrupted chamber immediately after the emplacement of silicic magma along the fractures (heavily dashed lines) shown in *a*. The depth of silicic magma in *b* is fixed by the lowest occurrence of silicic cumulates along the walls (as shown by Fig. 1). *c*, State of the chamber after subsequent replenishment by basic magma.

have been generated locally by fractional crystallization or melting of the chamber margins. Instead, it must have been emplaced from depth, possibly rapidly, into a magma chamber floored by a more basic magma. Magma chamber expansion due to rapid rise of silicic magmas into very shallow-level basic magma chambers may disrupt the roof and lead to explosive silicic volcanism.

Basic magma chambers make ideal passageways for silicic magmas<sup>13</sup>, and the nearly universal control of basaltic compositions by low-pressure equilibria suggests that shallow chambers are present in all tectonic environments. Where basic magma passes through the continental crust, silicic magmas will probably be generated by crustal melting; these silicic magmas should continue to rise from the lower crust after basic magma chambers have been established in the upper crust. They will tend to rise along existing structural discontinuities and pre-heated passageways and are therefore likely to encounter chambers of basic magma in the upper crust. Because of the higher density and temperature of basic magma, silicic magmas will move rapidly to the top of any chamber, acquiring both heat and kinetic energy for further eruption. Any connecting silicic chambers or passageways that lie beneath the basic magma chamber should be tapped rapidly and efficiently and could result in crustal readjustments similar to that shown by the NILI. Entry of silicic magma into a basic magma chamber provides an ideal opportunity to superheat the silicic magma<sup>1</sup>, as it can acquire heat both from the basic magma and from the pre-heated chamber margins.

Emplacement of silicic magma into a basic magma chamber is more likely to promote extensive commingling than if basic magma is emplaced into silicic: the density contrast of the two magmas guarantees rapid movement of silicic magma through the basic magma and extensive mutual contact of the most contrasted magmas, even if the magma chamber is compositionally stratified. Very little mixing may result when basic magmas are injected into silicic chambers<sup>14</sup>; if the chamber is compositionally stratified, there can be little chance of mixing between highly contrasted magmas.

Sparks *et al.*<sup>1</sup> recognized that the injection of basic magma into a silicic chamber would provide an effective mechanism for triggering acid explosive eruptions. Because more energy is efficiently transferred to the silicic magma, the opposite sequence, as recognized in the NILI, provides an even more effective setting in which to trigger explosive eruptions and can more readily account for commingling between highly contrasted magmas just before eruption.

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## Dutch elm disease as an analogue of Neolithic elm decline

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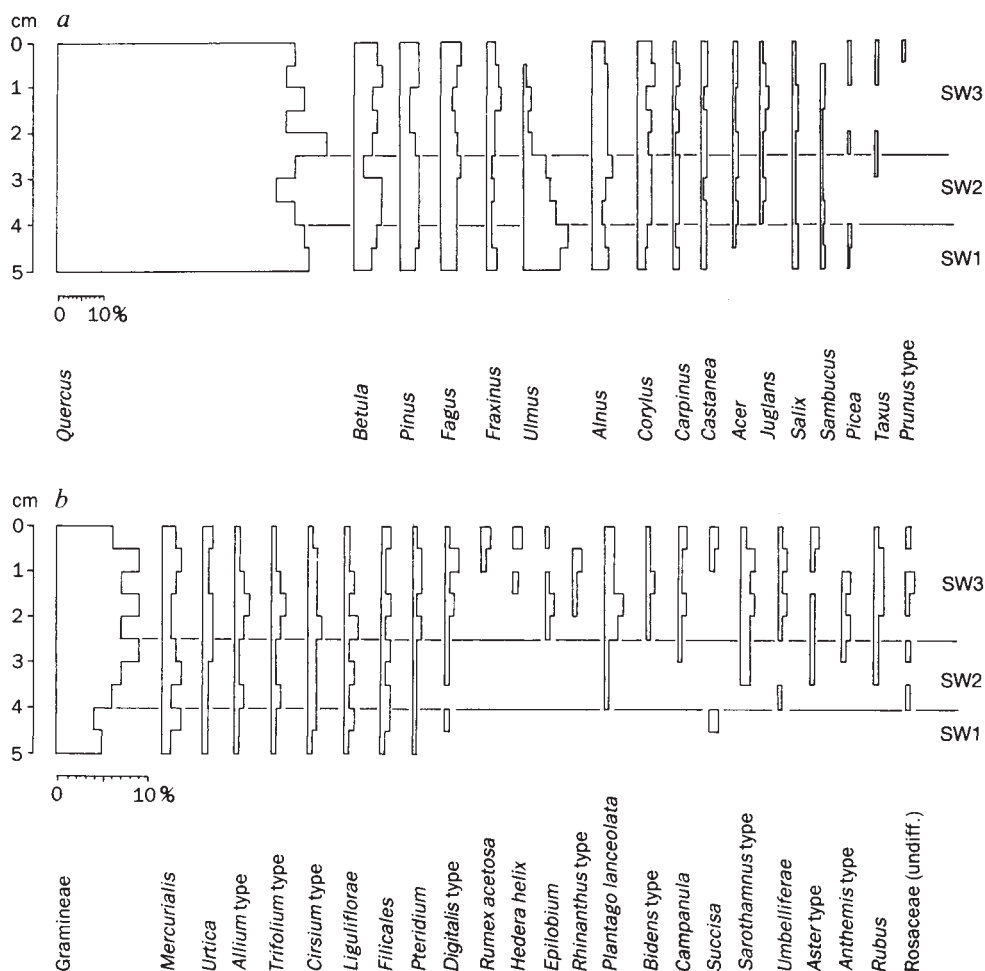
The decline in elm pollen about 5,000 radiocarbon years ago, recorded in the lake sediments and peats of north-west Europe<sup>1</sup>, has long been a source of speculation for palaeoecologists. Initially it was explained as a consequence of climatic change or degrading soils<sup>2</sup>. Later it was put down to the selective removal of elm branches by Neolithic cultures for use as fodder for animals kept in stalls<sup>3-5</sup>. An alternative explanation involves disease<sup>6-8</sup> and this possibility has received increasing support as a result of the recent effects of *Ceratocystis* on elms in Europe<sup>9</sup>. In North America, Davis<sup>10</sup> has examined the possibility of using recent evidence from the decline of *Castanea* this century<sup>11,12</sup> to interpret the decline in *Tsuga* pollen 4,800 years ago. In Europe, the current elm disease has provided an opportunity to observe the effects of the population decline of elms upon the pollen record. Pollen analysis of humus profiles from a woodland once rich in elm show an elm pollen decline of similar proportions to that found in Neolithic levels and also show an increased input of pollen from weed species as a result of the opening of the woodland canopy.

Much of the Lower Greensand ridge running parallel with and to the south of the North Downs in Kent is covered with mixed deciduous forest. At Scords Wood near Westerham (grid reference TQ478522), the oak-dominated woodland of the escarpment was rich in elm (*Ulmus minor* Miller) on the lower

slopes. Before the arrival of elm disease at the site in 1978 the pollen rain from this mixed forest consisted of between 5% and 60% *Ulmus* pollen depending upon the local forest composition. All adult trees of elm were affected by the disease and had been killed within about four years. In late 1985, the raw surface humus of the forest floor was sampled by removing successive layers of organic matter 0.5 cm thick. From these the pollen was extracted by conventional methods<sup>13</sup> and the results are shown in Fig. 1.

The diagram is divided into three zones on the basis of the elm pollen content. In the basal zone, SW1, elm pollen levels are in excess of 10%, reflecting conditions before the incidence of elm disease in 1978. There follows a zone in which elm pollen falls progressively, denoting the advancement of the disease and a consequent reduction in flowering even while the trees still lived. Finally, in zone SW3 the elm pollen dies away altogether.

The pollen proportions of the other tree and shrub species (Fig. 1a) are unaffected by the elm decline, as are some local woodland types, such as *Mercurialis*, *Urtica* and *Filicales* (Fig. 1b). But several others show an increase during or after the elm fall. Some of these, such as *Allium* and *Rubus*, are also local species of the woodland floor and their increased pollen input is likely to be a consequence of more prolific flowering following the opening of the tree canopy. But many others, such as *Plantago lanceolata*, *Bidens* type and *Campanula* are not present in the woodland and must be entering the pollen rain from more distant habitats. This has occurred because the more open tree canopy has permitted a better dispersal of these pollen types. Overall, there is a marked increase in the richness and diversity of the pollen assemblage after the elm decline. The mean number of pollen taxa recorded in each zone rises from 24 in SW1, through 27 in SW2, to 35 in SW3.



**Fig. 1** Pollen diagram from the upper layers of mor humus in deciduous woodland at Scords Wood, Kent. *a*, Tree species; *b*, other species. Data are expressed as percentage of total pollen. The boundary between zones SW1 and SW2 is considered to correspond with the arrival of Dutch elm disease at the site in 1978.



These results are of considerable interest for the interpretation of the Neolithic elm decline. First, they show that the death of elm trees by disease results in a very similar proportional reduction in elm pollen to that found in fossil samples. Second, they indicate that the increased diversity of fossil pollen types at the time of the elm decline, particularly those types often associated with human disturbance, such as *Plantago lanceolata*, may come about as a result of the alteration of those physical conditions, such as tree canopy structure, which previously restricted their pollen dispersal.

That weed pollen first comes into prominence in fossil pollen diagrams at the Neolithic elm decline could therefore be a consequence of the loss of elm trees and the resulting general effect on pollen dispersion. Evidence for forest clearance and cultivation before the elm decline is becoming increasingly strong and fits in well with this hypothesis<sup>14,15</sup>. This possibility also weakens the case for a man-induced elm decline, which is based on just such circumstantial evidence. The argument for a disease-induced elm decline in Neolithic times (once strongly rejected by Heybroek<sup>16</sup>) has been strengthened by the historical and dendrochronological work of Rackham. It has also been given greater credence by the recent finding of the beetle vector in Neolithic deposits in Hampstead, London<sup>17</sup>, although one

beetle does not prove the existence of the disease. The data presented here, however, show that an elm disease could generate pollen changes consistent with those observed in fossil pollen diagrams.

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## Ligand binding to the $\beta$ -adrenergic receptor involves its rhodopsin-like core

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Recently the genes for several hormone receptors that interact with guanine nucleotide binding proteins (G proteins) have been cloned, including the hamster  $\beta_2$ -adrenergic receptor ( $\beta_2AR$ )<sup>1</sup>, a human  $\beta AR$ <sup>2</sup>, the turkey erythrocyte  $\beta AR$ <sup>3</sup> and the porcine muscarinic acetylcholine receptor (MAR)<sup>4</sup>. All these receptors share some amino-acid homology with rhodopsin, particularly in 7 hydrophobic stretches of residues that are believed to represent transmembrane helices<sup>5</sup>. To determine whether differences in ligand specificity result from the divergence in the sequences of the hydrophilic regions of these receptors, we have expressed in mammalian cells genes for the wild-type hamster and human  $\beta AR$  proteins, and a series of deletion mutant genes of the hamster  $\beta_2AR$ . The pharmacology of the expressed receptors indicates that most of the hydrophilic residues are not directly involved in the binding of agonists or antagonists to the receptor. In addition, we have identified a mutant receptor that has high agonist affinity but does not couple to adenylate cyclase.

In Fig. 1 the amino-acid sequences of the hamster  $\beta_2AR$ , a human  $\beta AR$  and the turkey  $\beta AR$ , all of which bind catecholamines, are compared with those of the porcine MAR and bovine opsin, which interact with acetylcholine and retinal, respectively. The hamster  $\beta_2AR$  and the turkey  $\beta AR$  both couple to adenylate cyclase but differ in their specificity towards catecholamines<sup>6</sup>; the hamster  $\beta_2AR$  being of the  $\beta_2$ -subclass and the turkey  $\beta AR$  of the  $\beta_1$ -subclass. Previously, the pharmacology of the human receptor was unknown. Each group of receptors modulates different intracellular second messengers through interactions with specific G proteins;  $G_s$  for the  $\beta AR$ s,  $G_o$  and  $G_i$  for MAR<sup>7</sup> and transducin for opsin<sup>8</sup>. The different receptors vary in amino-acid sequence mostly in their C-terminal

regions and their putative third intracellular segments. To investigate the role of these hydrophilic regions in the binding of ligands by the hamster  $\beta_2AR$ , we constructed the series of deletion mutants listed in Table 1.

The genes for the hamster  $\beta_2AR$ , its human homologue and the deletion mutants were inserted into the SV40-derived vector pSVL as described in Fig. 2, legend. Transfection of the expression vector clones containing either the human or the hamster  $\beta AR$  cDNAs into COS-7 (ref. 9) cells resulted in an increase in the specific binding of the  $\beta$ -adrenergic antagonist iodocyanopindolol (<sup>125</sup>I-CYP) to plasma membranes prepared from the cells (Fig. 2, legend). The expressed  $\beta AR$ s displayed affinities for <sup>125</sup>I-CYP of 30-50 pM (see Table 1), approximating that of the naturally occurring  $\beta_2AR$  in control COS-7 cells and equivalent to that reported for purified receptor<sup>6,10</sup>. Agonist competition binding experiments (Fig. 2a) revealed that the  $\beta$ -adrenergic ligand binding sites resulting from expression of either the hamster or the human gene were indeed of the  $\beta_2$ -subtype, with the relative affinities for agonists being isoprenaline > adrenaline > noradrenaline, in agreement with published values<sup>10</sup>.

The expression of the genes encoding the mutant  $\beta AR$ s resulted in increases in both <sup>125</sup>I-CYP and isoprenaline binding sites (see Table 1). All the expressed mutant receptors were capable of binding <sup>125</sup>I-CYP with wild-type affinities with the exception of HAM(del 274-330) which lacked binding and HAM(del 179-187) which bound antagonist and agonist with reduced affinity. HAM(del 274-330) lacks the region encompassing the transmembrane helices VI and VII and HAM(del 179-187) has a deletion within the third external segment. A deletion at the N terminus, HAM(del 21-30), removal of 65 C-terminal residues, HAM(trun 354), and elimination of the third intracellular region, HAM(del 229-236) and HAM(del 239-272), did not dramatically alter ligand binding.

Membrane proteins were electrophoresed and immunoblotted with antisera raised against a peptide corresponding to the C-terminal sequence of the hamster  $\beta_2AR$  protein (Fig. 2, legend). For cells transfected with the wild-type hamster  $\beta_2AR$  gene (plasmid pSVHAM) a strongly reactive band with an apparent relative molecular mass of 67,000 ( $M_r$  67K) was observed which comigrated with purified  $\beta_2AR$  (Fig. 2b). A similar immunoreactive band was barely visible for cells transfected with the plasmid pSVL alone, corresponding to the endogenous COS-7 cell  $\beta_2AR$ . In addition to the 67K band,

Table 1 Ligand binding to mutant  $\beta_2$ AR proteins

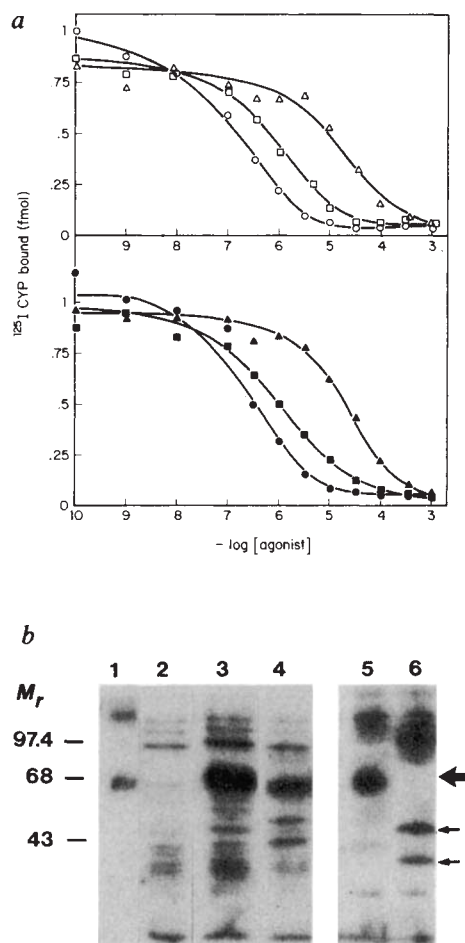
| Protein          | Region | COS-7 cells                           |              |                                       | L cells      |                   |                      |                |             |
|------------------|--------|---------------------------------------|--------------|---------------------------------------|--------------|-------------------|----------------------|----------------|-------------|
|                  |        | $^{125}$ I-CYP                        | Isoprenaline | $^{125}$ I-CYP                        | Isoprenaline | Adenylate cyclase |                      |                |             |
|                  |        | $B_{max}$<br>(fmol mg <sup>-1</sup> ) | $K_D$ (pM)   | $B_{max}$<br>(fmol mg <sup>-1</sup> ) | $K_D$ (pM)   | $R_H:R_L$         | $K_D(H):K_D(L)$ (nM) | $K_{act}$ (nM) | Stimulation |
| Control          |        | 48                                    | 20           | ND                                    | 0            | —                 | —                    | —              | 0 (5)       |
| HUM              |        | 2,000                                 | 50           | 300                                   | ND           | ND                | ND                   | ND             | ND          |
| HAM              |        | 3,300                                 | 45           | 300                                   | 110          | 70                | 55:45                | 1:50           | 20          |
| HAM(del 21-30)   | O1 (N) | 2,200                                 | 60           | 300                                   | 510          | ND                | 45:55                | 7:160          | 10          |
| HAM(del 179-187) | O3     | 4,400                                 | 600          | 10,000                                | 610          | 430               | 55:45                | 60:7,100       | 220         |
| HAM(del 229-236) | i3     | 3,100                                 | 60           | 100                                   | 840          | ND                | 60:40                | 8:190          | 5           |
| HAM(del 239-272) | i3     | 3,100                                 | 30           | 10                                    | 200          | 10                | 100:0                | 6              | —           |
| HAM(del 250-259) | i3     | 1,600                                 | ND           | ND                                    | 180          | ND                | 70:30                | 3:190          | 30          |
| HAM(del 259-262) | i3     | 4,100                                 | ND           | ND                                    | 130          | ND                | 70:30                | 1:190          | 50          |
| HAM(del 274-330) | VI-VII | 70                                    | ND           | ND                                    | ND           | ND                | ND                   | ND             | ND          |
| HAM(del 343-348) | i4     | 3,000                                 | ND           | ND                                    | 73           | ND                | 55:45                | 1:70           | 40          |
| HAM(trun 354)    | i4 (C) | 1,300                                 | 50           | 300                                   | 350          | 150               | 70:30                | 2:210          | 10          |

Membranes were prepared from COS-7 cells and L cells expressing wild-type or mutant  $\beta_2$ AR, and ligand-binding and adenylate cyclase assays were performed as in Figs 2 and 3. Names of mutant proteins are as in Fig. 2, and of the regions deleted as in Fig. 1. Values of  $K_D$  were determined for  $^{125}$ I-CYP binding by Scatchard analysis<sup>17</sup> based on a single class of binding sites. Where no  $K_D$  is shown, the density of binding sites was determined in the presence of a single concentration of 225 pM  $^{125}$ I-CYP. In all cases, nonspecific binding was <5%. Measurements of isoprenaline binding and of adenylate cyclase stimulation were as in Fig. 3. For COS-7 cell membranes EC<sub>50</sub> values were obtained by visual inspection of binding isotherms. For L cells, competition binding data were analysed using the LIGAND nonlinear regression programs of Munson and Rodard<sup>18</sup>. Results are reported for two affinity states of the receptor only in cases where a two-site fit was significantly better than a one-site fit ( $P < 0.01$ ). Adenylate cyclase stimulation parameters were determined by nonlinear regression analysis. The fold-stimulation by adenylate cyclase is given as a range in cases where more than one clonal isolate expressing the same mutant  $\beta_2$ AR was studied. The number in parentheses indicates the number of clonal isolates of each mutant characterized. All data shown are the means of 1-5 experiments, each done in triplicate. Standard errors were <15%. Abbreviations: N, N-terminus; C, C-terminus; ND, not determined; EC<sub>50</sub>, value at 50% maximal binding;  $B_{max}$ , saturation value for  $^{125}$ I-CYP binding to membranes;  $R_H:R_L$ , ratio of receptors in the high- and low-affinity states.

|        |             | O1           |               | I          |           |                     |               |
|--------|-------------|--------------|---------------|------------|-----------|---------------------|---------------|
| HAMBAR | MG          | PP           | NDSD          | FLLITNGSH  | VPI       | DHDVTEERDEAVVVGMAIL | MSVIVLAIVFG   |
| HUMBAR | MG          | PP           | NGSA          | FLLAPNRSH  | AP        | DHDVTQDEAVVVGMAIL   | MSLIVLAIVFG   |
| TURBAR | MG          | PP           | NDSD          | FLLITNGSH  | VPI       | DHDVTQDEAVVVGMAIL   | MSLIVLAIVFG   |
| PORMAR | M           | NTSAPP       | AYSPNITVL     | AP         | GKG       | PWQVAFIGIT          | TTGILLSLATVIG |
| BOVOPS | M           | NGTEGPNF     | VYFSPNITVL    | GVRSP      | FEAPQYVLA | EPWQFSMLAAV         | MFLLTLLG      |
|        |             | II           |               | O2         |           | III                 |               |
| HAMBAR | NVLVITAI    | AKFERLQTV    | NYFITSLACADLV | MGLAVVFGA  | SHLMKMW   | FGNFWCEFWTS         | IDVLCVTA      |
| HUMBAR | NVLVITAI    | AKFERLQTV    | NYFITSLACADLV | MGLAVVFGA  | SHLMKMW   | FGNFWCEFWTS         | IDVLCVTA      |
| TURBAR | NVLVITAI    | AKFERLQTV    | NYFITSLACADLV | MGLAVVFGA  | SHLMKMW   | FGNFWCEFWTS         | IDVLCVTA      |
| PORMAR | NVLVITAI    | AKFERLQTV    | NYFITSLACADLV | MGLAVVFGA  | SHLMKMW   | FGNFWCEFWTS         | IDVLCVTA      |
| BOVOPS | NVLVITAI    | AKFERLQTV    | NYFITSLACADLV | MGLAVVFGA  | SHLMKMW   | FGNFWCEFWTS         | IDVLCVTA      |
|        |             | IV           |               | O3         |           |                     |               |
| HAMBAR | SIETLCVIA   | VDYIAITSP    | FKYQSLLTK     | NKARV      | IVMVV     | VSGLTSFLP           | IQMHWRATHQK   |
| HUMBAR | SIETLCVIA   | VDYIAITSP    | FKYQSLLTK     | NKARV      | IVMVV     | VSGLTSFLP           | IQMHWRATHQK   |
| TURBAR | SIETLCVIA   | VDYIAITSP    | FKYQSLLTK     | NKARV      | IVMVV     | VSGLTSFLP           | IQMHWRATHQK   |
| PORMAR | SIETLCVIA   | VDYIAITSP    | FKYQSLLTK     | NKARV      | IVMVV     | VSGLTSFLP           | IQMHWRATHQK   |
| BOVOPS | SIETLCVIA   | VDYIAITSP    | FKYQSLLTK     | NKARV      | IVMVV     | VSGLTSFLP           | IQMHWRATHQK   |
|        |             | V            |               |            |           |                     |               |
| HAMBAR | HKE         | TCCDFFTNQ    | AYIA          | SSIVSFYVPL | VYVYYSR   | VFOQVAK             | RQLQKIDKSE    |
| HUMBAR | HKE         | TCCDFFTNQ    | AYIA          | SSIVSFYVPL | VYVYYSR   | VFOQVAK             | RQLQKIDKSE    |
| TURBAR | HKE         | TCCDFFTNQ    | AYIA          | SSIVSFYVPL | VYVYYSR   | VFOQVAK             | RQLQKIDKSE    |
| PORMAR | HKE         | TCCDFFTNQ    | AYIA          | SSIVSFYVPL | VYVYYSR   | VFOQVAK             | RQLQKIDKSE    |
| BOVOPS | HKE         | TCCDFFTNQ    | AYIA          | SSIVSFYVPL | VYVYYSR   | VFOQVAK             | RQLQKIDKSE    |
|        |             | VI           |               |            |           |                     |               |
| HAMBAR | GRFHS       | PNLGQVEQDGRS | GHG           | L          | RRSSK     | FCL                 | KEHKALKTLGIIM |
| HUMBAR | GRFHS       | PNLGQVEQDGRS | GHG           | L          | RRSSK     | FCL                 | KEHKALKTLGIIM |
| TURBAR | GRFHS       | PNLGQVEQDGRS | GHG           | L          | RRSSK     | FCL                 | KEHKALKTLGIIM |
| PORMAR | GRFHS       | PNLGQVEQDGRS | GHG           | L          | RRSSK     | FCL                 | KEHKALKTLGIIM |
| BOVOPS | GRFHS       | PNLGQVEQDGRS | GHG           | L          | RRSSK     | FCL                 | KEHKALKTLGIIM |
|        |             | VII          |               |            |           |                     |               |
| HAMBAR | WLPFFIVNI   | VHVIO        | DNLIPKEVY     | ILLNWLG    | YVNSA     | FNPLIYC             | RSPDFRIAF     |
| HUMBAR | WLPFFIVNI   | VHVIO        | DNLIPKEVY     | ILLNWLG    | YVNSA     | FNPLIYC             | RSPDFRIAF     |
| TURBAR | WLPFFIVNI   | VHVIO        | DNLIPKEVY     | ILLNWLG    | YVNSA     | FNPLIYC             | RSPDFRIAF     |
| PORMAR | WLPFFIVNI   | VHVIO        | DNLIPKEVY     | ILLNWLG    | YVNSA     | FNPLIYC             | RSPDFRIAF     |
| BOVOPS | WLPFFIVNI   | VHVIO        | DNLIPKEVY     | ILLNWLG    | YVNSA     | FNPLIYC             | RSPDFRIAF     |
|        |             | O4           |               |            |           |                     |               |
| HAMBAR | WLPFFIVNI   | VHVIO        | DNLIPKEVY     | ILLNWLG    | YVNSA     | FNPLIYC             | RSPDFRIAF     |
| HUMBAR | WLPFFIVNI   | VHVIO        | DNLIPKEVY     | ILLNWLG    | YVNSA     | FNPLIYC             | RSPDFRIAF     |
| TURBAR | WLPFFIVNI   | VHVIO        | DNLIPKEVY     | ILLNWLG    | YVNSA     | FNPLIYC             | RSPDFRIAF     |
| PORMAR | WLPFFIVNI   | VHVIO        | DNLIPKEVY     | ILLNWLG    | YVNSA     | FNPLIYC             | RSPDFRIAF     |
| BOVOPS | WLPFFIVNI   | VHVIO        | DNLIPKEVY     | ILLNWLG    | YVNSA     | FNPLIYC             | RSPDFRIAF     |
|        |             | i4           |               |            |           |                     |               |
| HAMBAR | KAYGNGYSSNS | GKTDYM       | GEASQ         | COLG       | QEKESER   | LCED                | PGTESFVNC     |
| HUMBAR | KAYGNGYSSNS | GKTDYM       | GEASQ         | COLG       | QEKESER   | LCED                | PGTESFVNC     |
| TURBAR | KAYGNGYSSNS | GKTDYM       | GEASQ         | COLG       | QEKESER   | LCED                | PGTESFVNC     |
| PORMAR | KAYGNGYSSNS | GKTDYM       | GEASQ         | COLG       | QEKESER   | LCED                | PGTESFVNC     |
| BOVOPS | KAYGNGYSSNS | GKTDYM       | GEASQ         | COLG       | QEKESER   | LCED                | PGTESFVNC     |

Fig. 1 Comparison of amino-acid sequences for G-protein coupled hormone receptors and rhodopsin. The amino-acid sequences for the hamster  $\beta_2$ -adrenergic receptor (HAMBAR)<sup>1</sup>, human  $\beta$ -adrenergic receptor (HUMBAR)<sup>2</sup>, turkey  $\beta$ -adrenergic receptor (TURBAR)<sup>3</sup>, porcine cerebral muscarinic acetylcholine receptor (PORMAR)<sup>4</sup>, and bovine opsin (BOVOPS)<sup>5</sup> are shown. The entire sequence for each receptor is shown with gaps (·) introduced to obtain the best alignment. Amino acids have been aligned to display regions of maximum homology among all the sequences, with identities indicated by boxes around individual amino acids. Regions I-VII correspond to hydrophobic residues postulated to be transmembrane helices. Based on the rhodopsin model the hydrophilic residues between each transmembrane helix are designated extracellular (O) or intracellular (i) to indicate the proposed orientation of each segment of the protein with respect to the membrane<sup>19</sup>.





**Fig. 2** Expression of wild-type and mutant  $\beta_2\text{AR}$  in COS-7 cells. **a**, Competition for the binding of  $^{125}\text{I}$ -CYP by (-)isoprenaline (O), (-)adrenaline (□), and (-)noradrenaline (Δ) to membranes from COS-7 cells transfected with pSVHAM (open symbols, above) or pSVHUM (closed symbols, below). Total  $^{125}\text{I}$ -CYP binding at saturation was 3,300 fmol per mg protein (pSVHAM) and 2,000 fmol mg $^{-1}$  (pSVHUM). Control COS-7 cells transfected with pSVL had 48 fmol per mg protein  $^{125}\text{I}$ -CYP binding sites. The data shown are a representative example of three separate experiments, each point performed in duplicate. Nonspecific binding of  $^{125}\text{I}$ -CYP in the presence of 10  $\mu\text{M}$  alprenolol was <5%. **b**, Protein immunoblot of  $\beta_2\text{AR}$ -related proteins expressed in cells transfected with pSVL (lanes 2), pSVHAM (lanes 3, 5), pSVHAM(del 239-272) (lane 4) and pSVHAM(del 274-330) (lane 6). Lane 1 contains purified hamster lung  $\beta_2\text{AR}$ . Numbers at left, size standards in thousands; large arrow, position of wild-type  $\beta_2\text{AR}$ ; small arrows, positions of mutant proteins.

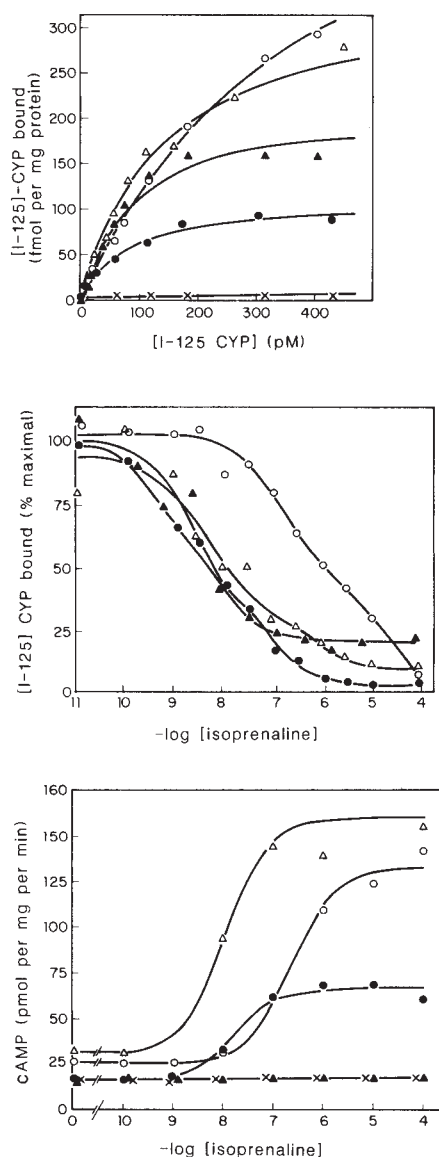
**Methods.** Mutations were introduced into the hamster  $\beta_2\text{AR}$  gene (contained in pSVHAM) by oligonucleotide-directed mutagenesis<sup>20</sup>. Deletions were made using oligonucleotides 30 nucleotides (nt) in length containing sequences evenly flanking the desired deletion. These mutants are designated as pSVHAM(del A-B), where A and B are the first and last amino acid of the hamster  $\beta_2\text{AR}$  sequence included in the deleted region. Single base substitutions were introduced using appropriate mismatched oligonucleotides 16-20 nt in length. The mutant HAM(trun 354) contains a single base substitution converting Tyr 354 to a termination codon. Authenticity was confirmed by dideoxy sequencing<sup>21</sup>. The plasmid pSVL contains the SV40 early promoter and the 16S intron derived from the Okayama-Berg vector pL<sup>22</sup> cloned<sup>23</sup> into the *Hind*III and *Pst*I sites of pUC13. The hamster  $\beta_2\text{AR}$  cDNA and the deletion mutants were cloned between the *Sma*I and *Eco*RI sites of pSVL as a 1.9-kb fragment originating at the *Eag*I site 86 bp 5' to the initiator Met and extending 23 bp 3' to the polyadenylation signal, giving pSVHAM. Similarly, cDNA for the human  $\beta_2\text{AR}$  was cloned into the same sites of pSVL as a 1.8-kb fragment beginning at the *Nco*I site corresponding to the initial Met and encompassing the polyadenylation signal to give pSVHUM. Transfection of plasmids into COS-7 cells was carried out using the CaPO<sub>4</sub> precipitation method<sup>24</sup>. Plasmid DNA (15  $\mu\text{g}$ ) was mixed with 15  $\mu\text{g}$  of calf thymus DNA and precipitated with 125 mM CaCl<sub>2</sub>. Precipitates were applied to 10<sup>6</sup> COS-7 cells and incubated at 37 °C for 4 h. Cells were glycerol shocked and incubated in fresh media for 72 h, at which time they were collected by scraping and washing once with phosphate-buffered saline. For a rapid determination of ligand-binding properties of various mutants, COS-7 cells expressing the receptor were lysed by freeze-thawing in 15 mM Tris, pH 7.5, 2 mM MgCl<sub>2</sub>, 0.3 mM EDTA. Membranes were pelleted by centrifugation at 40,000g and resuspended at 1-3 mg protein per ml in 75 mM Tris, pH 7.5, 12.5 mM MgCl<sub>2</sub>, 0.3 M EDTA. Saturation binding of  $^{125}\text{I}$ -CYP (NEN) was measured at a final protein concentration of 20  $\mu\text{g}$  ml $^{-1}$  using 10-400 pM  $^{125}\text{I}$ -CYP, with nonspecific binding defined in the presence of 10  $\mu\text{M}$  alprenolol<sup>25</sup>. Isoprenaline binding was measured in competition with 35 pM  $^{125}\text{I}$ -CYP, at a final  $\beta_2\text{AR}$  concentration of 5-7 pM, as described<sup>25</sup>. Protein concentrations were determined by the method of Lowry<sup>26</sup>. Protein immunoblot analysis of the membranes was performed as described<sup>1</sup>. Polyclonal antisera raised against a peptide corresponding to the C terminus of the hamster  $\beta_2\text{AR}$  protein was used to detect the  $\beta_2\text{AR}$ -specific polypeptides. A peptide having the sequence CLDSQGRN(nor-leucine)STNDSPL (amino-acid residues 404-418 of the hamster  $\beta_2\text{AR}$ ) was synthesized using an Applied Biosystems peptide synthesizer and purified by reversed phase HPLC. This peptide was coupled through its N-terminal cysteine to thyroglobulin (R. Mumford and C.D.S., in preparation) and used as an immunogen in rabbits. Total rabbit serum was used as the first antibody in the immunoblot at a 1:500 dilution followed by detection with  $^{125}\text{I}$ -protein A as described<sup>1</sup>. Purification of  $\beta_2\text{AR}$  was described<sup>10</sup>.

specifically immunoreactive proteins with apparent  $M_r$  46K and 55-58K were also observed in the cells transfected with pSVHAM, presumably representing nonglycosylated and partially glycosylated  $\beta_2\text{AR}$  proteins, respectively<sup>10</sup>. Immunoreactive bands of higher apparent  $M_r$  were occasionally detected and probably represent aggregates of the receptor. HAM(del 239-272), which bound ligand, was expressed at levels similar to that of the wild-type receptor and had the expected  $M_r$ . For HAM(del 274-330), which did not bind ligand, immunoreactive peptides of lower  $M_r$  were observed. The  $M_r$ s of the peptides were consistent with both a deletion in the proteins and a defect in their glycosylation. Whether the lack of ligand binding for this mutant receptor is due to the loss of sequences directly involved in ligand binding or to a defect in protein folding and glycosylation is unknown.

Although the transient expression of the  $\beta_2\text{AR}$  genes in COS-7 cells permitted the binding properties of the expressed proteins to be measured rapidly, with minimal selection, the low frequency of expressing cells in combination with the  $\beta_2\text{AR}$  background in this cell line prevented characterization of the coupling of the receptor with the adenylate cyclase system.

Consequently, clonal lines of mouse L cells<sup>11</sup> stably expressing the wild-type receptor and mutants which exhibited ligand binding in COS-7 cells, were established by the procedure described in Fig. 3 legend. Mouse L cells were chosen because they have undetectable levels of  $\beta_2\text{AR}$  as assessed by ligand binding (Fig. 3a) and they lack a  $\beta$ -adrenergic-stimulated adenylate cyclase, but contain measurable levels of both prostaglandin E<sub>1</sub>- and NaF<sup>12</sup>-stimulated adenylate cyclase activity (data not shown). The density of receptor sites for each of the cell lines was measured by  $^{125}\text{I}$ -CYP binding (Table 1). Because L cells lack an endogenous  $\beta_2\text{AR}$  background, the  $K_D$  values for  $^{125}\text{I}$ -CYP and isoprenaline binding to each of the cell lines could be calculated. The affinities for  $^{125}\text{I}$ -CYP of wild-type and mutant hamster  $\beta_2\text{AR}$  proteins expressed in L cells were similar to those observed in COS-7 cells (Fig. 3a and Table 1). In these cells, HAM(del 239-272) had a slightly greater affinity for  $^{125}\text{I}$ -CYP than the wild-type receptor and mutant HAM(del 179-187) exhibited the same reduced affinity for  $^{125}\text{I}$ -CYP as observed for the COS-7 cells (Fig. 3a).

Data for isoprenaline competition binding and stimulation of adenylate cyclase in membranes containing the wild-type and



**Fig. 3** Saturation binding of [<sup>125</sup>I]-CYP (a), isoprenaline competition for [<sup>125</sup>I]-CYP binding (b), and isoprenaline stimulation of adenylyl cyclase (c) in membranes from L cell lines containing pSVL (x), pSVHAM (●), pSVHAM(del 179-187) (○), pSVHAM(del 239-272) (▲), and pSVHAM(trun 354) (△). Adenylyl cyclase activity (pmol cAMP per mg per min) in the same membranes stimulated by 10 mM NaF was 46 for pSVL, 87 for pSVHAM, 124 for pSVHAM(del 179-187), 73 for pSVHAM(del 239-272), and 101 for pSVHAM(trun 354). Each experiment shown is a representative of 2-5 experiments for each clone.

**Methods.** To obtain L cell lines expressing the  $\beta$ AR, each plasmid was cotransfected with 5  $\mu$ g of pRSVneo<sup>27</sup> as described in Fig. 2. Plasmid-bearing cells were selected by the addition of 500  $\mu$ g ml<sup>-1</sup> Geneticin (gentamicin, G-418, Gibco) to the media 24 h posttransfection<sup>28</sup>. Geneticin-resistant colonies (12-24) were picked from each of two separate transfections and assayed for [<sup>125</sup>I]-CYP binding as in Table 1. In all experiments 30-70% of the geneticin-resistant clones co-expressed the  $\beta$ AR. Positive clones (4-10) for each mutant were characterized for ligand binding. Plasma membranes were prepared from L cells by hypotonic lysis in 1 mM Tris, pH 7.5. After centrifugation at 40,000g, the membranes were resuspended at 1-3 mg protein per ml in 75 mM Tris, pH 7.5, 12.5 mM MgCl<sub>2</sub>, 1.5 mM EDTA. Ligand-binding experiments were performed as described in Fig. 2. Adenylyl cyclase stimulation in the presence of isoprenaline or 10 mM NaF was measured as described elsewhere<sup>29</sup>.

several of the mutant receptors are shown in Fig. 3b and c. The wild-type receptor exhibited both high- and low-affinity binding sites for isoprenaline (see Table 1)<sup>13</sup>. The occurrence of two agonist affinity sites has been attributed to an equilibrium between free receptor and receptor complexed with G<sub>s</sub>. Membranes prepared from cells expressing the wild-type receptor also demonstrated a dose-dependent activation of adenylyl cyclase by isoprenaline and NaF, but membranes derived from the control cells showed stimulation of adenylyl cyclase only by NaF. Although the maximum stimulations of adenylyl cyclase by isoprenaline varied from 3- to 6-fold for different clonal isolates of each mutant, they were identical to the NaF stimulations in the same membranes (Fig. 3c) indicating that the expressed receptors were fully coupled to adenylyl cyclase<sup>13,14</sup>. In sharp contrast with the wild-type receptor and HAM(trun 354), HAM(del 239-372) containing membranes exhibited a single high-affinity isoprenaline binding site and did not show any stimulation of adenylyl cyclase by isoprenaline (Table 1 and Fig. 3). It is not known whether the affinity exhibited by this site reflects the  $K_D$  of the free mutant receptor or of an inactive complex between the receptor and G<sub>s</sub>. The lack of adenylyl cyclase coupling to this mutant receptor might be due to either the elimination of a site on the receptor for G-protein interaction or to the importance of the deleted region in maintaining an active conformation. HAM(del 179-187), which showed a reduced affinity for isoprenaline, also required higher levels of isoprenaline for cyclase stimulation (Table 1 and Fig. 3). The remaining mutants showed both high- and low-affinity states and stimulated adenylyl cyclase by 2-8-fold in an isoprenaline-dependent manner (Table 1). The variation in the absolute level of stimulation between different clonal isolates allows for only a qualitative analysis of receptor-cyclase coupling. Future experiments measuring the stimulation of the GTPase of G<sub>s</sub><sup>14</sup> or the effect of GTP analogues on hormone binding will be necessary to compare quantitatively the abilities of the mutant receptors to couple with G proteins.

The absence of a large hydrophilic domain involved in ligand binding implies that ligands interact either with a site located close to the membrane or within the hydrophobic core of the protein. This hypothesis is supported by the observation that in the case of the turkey  $\beta$ AR, an affinity label bound to the receptor can covalently react with associated glycolipids<sup>15</sup>. Previously we had proposed, on the basis of the homology between the hamster  $\beta_2$ AR and bovine opsin that the  $\beta$ -adrenergic ligands interact with the receptor in a manner analogous to that of retinal interacting with opsin<sup>1</sup>. In rhodopsin retinal binds within the hydrophobic transmembrane region of the protein rather than to external hydrophilic residues<sup>16</sup>. The results described here lend further support to this model.

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## The condensing vacuole of exocrine cells is more acidic than the mature secretory vesicle

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A number of intracellular, membrane-bound compartments in both the endocytic and exocytic pathways of eukaryotic cells have an acidic internal pH<sup>1</sup>. In endocrine cells, the mature secretory vesicle has an acidic pH; secretory vesicles isolated from exocrine cells, however, appear to have a neutral pH<sup>2</sup>. Recently we have used a newly developed immunocytochemical technique<sup>3</sup> to map low-pH compartments in insulin-secreting islet cells with the electron microscope and find that during the maturation of the secretory vesicle there is a progressive acidification of these vesicles that begins as soon as the *trans* Golgi condensing vacuoles form<sup>4</sup>. Now we have used this technique to examine two exocrine cells: the pancreatic acinar cell and the parotid serous cell. In both cell types, the *trans* Golgi condensing vacuoles are acidic and accumulate the low-pH probe to the same extent as condensing vacuoles of insulin-secreting islet cells. Unlike insulin-secreting cells, however, maturation of the granules is accompanied by a return of luminal pH to near neutrality. Therefore, although the pH of

storage granules in exocrine and endocrine cells is different, the pH of the condensing vacuoles in both cells is acidic.

DAMP (3-(2,4-dinitroanilino)-3'-amino-N-methylidipropylamine) accumulates in acidic compartments and can be localized in plastic-embedded tissue sections with an antibody against dinitrophenol (DNP)<sup>5</sup>. To visualize acidic compartments in exocrine pancreatic cells by indirect immunofluorescence, isolated acini were incubated with 30  $\mu$ M DAMP, fixed and embedded in Epon. Serial thick sections (0.5  $\mu$ m thick) were prepared; one section was incubated with anti-amylase serum to localize secretory granules and the adjacent section was incubated with anti-DNP immunoglobulin G (IgG) to localize acidic compartments. DAMP was located in vacuoles that were scattered in the cytoplasm of each cell (Fig. 1a). In the companion section that was processed to localize amylase, there were numerous brightly fluorescent granules clustered together in the apex of the cells (Fig. 1b). Comparing the two images reveals that most of the amylase-positive granules were not positive for DAMP. Only a few amylase-positive granules just above the nucleus contained DAMP. Therefore, only a subpopulation of the amylase-positive granules appeared to be acidic.

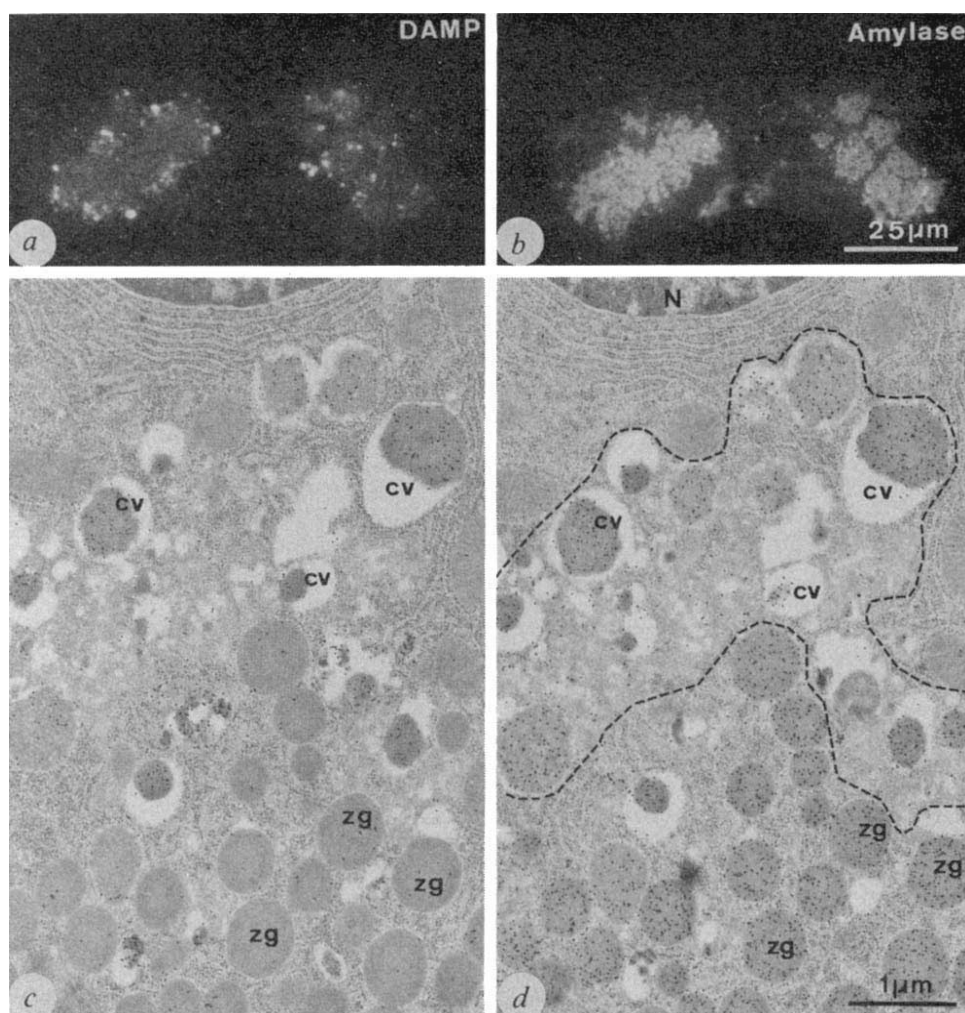
Both amylase and DAMP can be localized in Epon thin sections by an indirect protein A-gold procedure. Figure 1c and d shows the same juxtanuclear region of an exocrine cell from an acinus examined on adjacent serial thin section (80-100 nm thick) for the presence of DAMP (Fig. 1c) and amylase (Fig. 1d). The density of anti-amylase serum binding sites in the proteinaceous core of both condensing vacuoles and mature granules was the same. In contrast, only the proteinaceous core of the condensing vacuoles had a high density of anti-DNP IgG binding sites.

These qualitative results were confirmed by a quantitative analysis of the distribution of DAMP-specific gold particles in various cellular compartments of the exocrine cell (Table 1). The density of anti-DNP IgG binding sites over the mature secretory vesicles was nearly the same as the density over the nucleus, which indicates these vesicles were not acidic. In contrast, the density of gold particles in the condensing vacuoles was, on average, ninefold greater than in the mature vesicles. The density of DAMP-specific gold particles in lysosomes was higher than in the condensing vacuole. All anti-DNP IgG binding was abolished if the DAMP-treated acini were post-incubated in the presence of monensin (Table 1). Therefore, in these

Table 1 Density of monoclonal anti-DNP binding sites on pancreatic acinar cells

| Cellular compartment | Experiment 1 |         | Number of gold particles per $\mu$ m <sup>2</sup> of cell compartment |         |             |         | Experiment 3 |         |
|----------------------|--------------|---------|-----------------------------------------------------------------------|---------|-------------|---------|--------------|---------|
|                      |              |         | Experiment 2                                                          |         | Monensin    |         |              |         |
| Golgi stack          | 2 $\pm$ 1    | (21.58) | 3 $\pm$ 1                                                             | (18.41) | 1 $\pm$ 0.2 | (26.38) | 2 $\pm$ 1    | (17.87) |
|                      | n = 10       |         | n = 7                                                                 |         | n = 10      |         | n = 9        |         |
| Condensing vacuoles  | 55 $\pm$ 9   | (15.42) | 53 $\pm$ 12                                                           | (10.40) | 2 $\pm$ 0.6 | (16.29) | 60 $\pm$ 10  | (9.87)  |
|                      | n = 19       |         | n = 11                                                                |         | n = 10      |         | n = 11       |         |
| Granules             | 6 $\pm$ 1    | (19.13) | 6 $\pm$ 1                                                             | (15.02) | 2 $\pm$ 1   | (19.13) | 7 $\pm$ 1    | (15.57) |
|                      | n = 14       |         | n = 10                                                                |         | n = 10      |         | n = 10       |         |
| Lysosomes            | 111 $\pm$ 15 | (6.09)  | 74 $\pm$ 11                                                           | (4.86)  | 2 $\pm$ 1   | (8.93)  | 99 $\pm$ 14  | (10.76) |
|                      | n = 15       |         | n = 4                                                                 |         | n = 9       |         | n = 9        |         |
| Nucleus              | 2 $\pm$ 0.3  | (27.12) | 3 $\pm$ 1                                                             | (27.78) | 2 $\pm$ 0.3 | (36.21) | 1 $\pm$ 0.3  | (30.51) |
|                      | n = 7        |         | n = 5                                                                 |         | n = 7       |         | n = 5        |         |

Values are means  $\pm$  s.e.m.; n, no. of pictures evaluated. The no. of  $\mu$ m<sup>2</sup> evaluated for each compartment is shown in parentheses. Dispersed pancreatic acini, prepared as described<sup>14</sup> were incubated in a KRB-HEPES medium containing 0.1% bovine serum albumin (BSA) in the presence of 30  $\mu$ M DAMP for 30 min at 37  $^{\circ}$ C (experiment 1). In experiment 2, the cells were incubated an additional 30 min in the presence or absence of 25  $\mu$ M monensin. In experiment 3, acinar cells were incubated with DAMP for 2 h. At the end of each incubation, cells were rinsed, fixed for 2 h at room temperature with 1% glutaraldehyde buffered with 0.1 M sodium phosphate pH 7.4, dehydrated and embedded in Epon. Thin sections were collected on nickel grids and immunostained by the protein A-gold technique<sup>15</sup>. To localize DAMP, sections were incubated for 2 h with monoclonal anti-DNP antibody (0.1  $\mu$ g IgG ml<sup>-1</sup>) followed by rabbit anti-mouse IgG (12  $\mu$ g ml<sup>-1</sup>) and protein A-gold (1:70 v/v) for 1 h at room temperature. Sections were stained sequentially with uranyl acetate (20 min) and lead citrate (10 min). The distribution of DAMP in cellular compartments was quantified on electron micrographs at a calibrated magnification of 44,100. The number of gold particles and the areas of respective compartments were recorded with an electronic pen on a graphic tablet (Tektronix, type 4933) connected to a microcomputer (IBM PC) programmed to calculate the number of particles per  $\mu$ m<sup>2</sup> of the compartment.



**Fig. 1** *a,b*, Localization of DAMP (*a*) and amylase (*b*) by indirect immunofluorescence<sup>16</sup> in consecutive serial sections of two isolated pancreatic acini. *c,d*, Consecutive serial thin sections stained for DAMP (*c*) and amylase (*d*) with the protein A-gold technique (gold particles 15-nm diameter). Dotted line, Golgi region; N, nucleus. DAMP immunoreactivity is high over the secretory content of condensing vacuoles (cv) but not over the mature zymogen granules (zg). Both compartments are labelled with gold particles following treatment with anti-amylase serum. See Table 1 legend for the quantification of DAMP immunostaining and for immunocytochemical procedures.

**Methods.** In *b*, dispersed pancreatic acini were incubated for 30 min at 37 °C in the presence of 30  $\mu$ M DAMP as in Table 1. Cells were embedded in Epon. Sections collected on glass slides and processed to remove Epon<sup>17</sup> were incubated with anti-DNP IgG (5  $\mu$ g ml<sup>-1</sup>) or guinea pig anti-amylase serum (1:100 v/v) for 2 h and then exposed to FITC-conjugated anti-mouse or anti-guinea pig IgG for 1 h. DAMP immunofluorescence reveals sparse spots predominantly in the middle part of the acinar cell, whereas anti-amylase serum stains densely the centre of the acinus (apex of the cells).

exocrine cells only the condensing vacuoles and lysosomes were acidic.

We next examined the parotid acinar cell, an exocrine cell that has a secretory apparatus similar to the pancreatic exocrine cell. This cell also accumulated DAMP in condensing vacuoles (Fig. 2) but not in mature secretory vesicles. By quantitative analysis (Table 2), the density of DAMP-specific gold particles in condensing vacuoles and lysosomes was found to be similar to that in the same compartments of pancreatic acinar cells. Likewise, the Golgi cisternae and mature secretory vesicles did not have significant numbers of DAMP gold particles.

These two exocrine cells join the insulin-secreting endocrine cell<sup>4</sup> and the cultured human fibroblast<sup>5</sup> as cells that have been analysed with the DAMP technique. All four cell types have in common an acidic *trans* Golgi vesicular compartment. Yet the mature secretory vesicles derived from these condensing vacuoles can have either an acidic (endocrine cell) or neutral (exocrine cell) pH. Therefore, acidification of the *trans* Golgi condensing vacuoles must be functionally separate from the acidification of the mature secretory vesicle.

Most probably the acidic compartments of the Golgi apparatus have some function in the movement of proteins through the Golgi<sup>6</sup>, their post-translational modification<sup>7</sup> and their sorting into regulated and non-regulated exocytic pathways<sup>8</sup>. Drugs that disrupt pH gradients inhibit transit from the *medial* to the *trans* compartments of the Golgi apparatus<sup>9</sup> and shift proteins that are normally secreted by a regulated pathway into a non-regulated pathway<sup>10</sup>.

All the endocrine secretory vesicles examined to date have been found to have an acidic interior<sup>1</sup>, and the exocrine vesicles

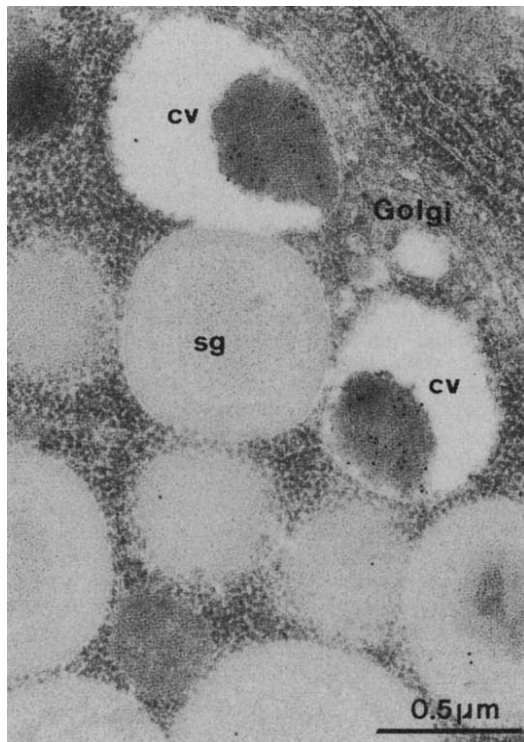
studied here are neutral. Exocrine cells have a conspicuous morphological and functional polarity; moreover, a number of physiological<sup>11</sup> and cell biological<sup>12</sup> studies suggest that endocrine cells also express a polarized organization. A fundamental difference between endocrine and exocrine cells is the compartment into which they release their secretory product. Whereas most endocrine cells secrete into an 'advascular' compartment, exocrine cells release their product into an 'abvascular' compartment (lumen of the gland). Perhaps the pH of the mature secretory vesicle directly or indirectly controls the polarity of movement towards the different surfaces of the cell: low-pH vesicles go to advascular surface and neutral-pH vesicles go to

**Table 2** Density of monoclonal anti-DNP binding sites on parotid acinar cells

| Cellular compartment                | No. of gold particles per $\mu$ m <sup>2</sup> |
|-------------------------------------|------------------------------------------------|
| Golgi stack                         | 2 $\pm$ 0.4 (26.90)<br><i>n</i> = 20           |
| Condensing vacuoles (core and halo) | 73 $\pm$ 12 (8.77)<br><i>n</i> = 16            |
| Granules                            | 2 $\pm$ 0.4 (39.27)<br><i>n</i> = 16           |
| Lysosomes                           | 159 $\pm$ 32 (3.23)<br><i>n</i> = 11           |
| Nucleus                             | 2 $\pm$ 0.3 (20.68)<br><i>n</i> = 7            |

Results are presented as in Table 1.





**Fig. 2** Localization of DAMP in thin sections of parotid exocrine cell. Gold particles (10 nm diameter) are numerous over condensating vacuoles (cv) but virtually absent over mature secretory granules (sg).

the opposite surface. In support of this proposal, Caplan *et al.*<sup>13</sup> recently reported that  $\text{NH}_4\text{Cl}$ , a weak base that raises the pH of intracellular compartments, abolishes the exclusive secretion of laminin and heparin sulphate proteoglycan from the basolateral surface of polarized MDCK cells.

The alkalinization of the maturing exocrine secretory vesicle clearly indicates that the pH of this compartment is regulated, perhaps by the removal of the proton pump from the vesicle membrane. Alternatively, the pump may be inactivated or a permeant anion channel may close<sup>1</sup>. All these possibilities will lead to the dissipation of the pH gradient in the condensing vacuole as it matures into a secretory vesicle. Future studies of the exocrine cell may lead to important insights into how pH is regulated in various intracellular compartments.

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## Differential expression of two distinct T-cell receptors during thymocyte development

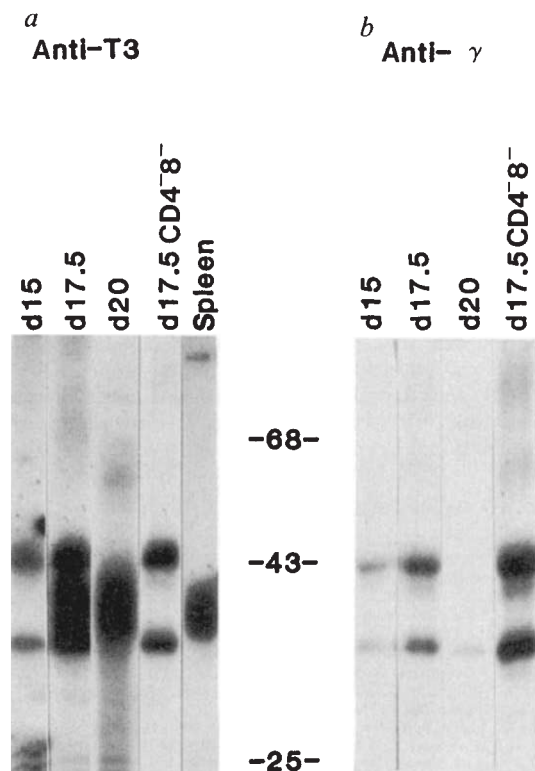
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The product of the T-cell receptor (TCR)  $\gamma$ -gene<sup>1</sup> has recently been found to be expressed on a subset of both peripheral cells<sup>2</sup> and thymocytes<sup>3,4</sup>. As an initial approach to understanding the role of this  $\gamma$ -chain of TCR (TCR $\gamma$ ) in T-cell development, we have studied the ontogeny of TCR expression at the protein level in the developing murine thymus. We show here that the first T3-associated TCR to be expressed in the developing thymus is a disulphide-linked heterodimer composed of a  $\gamma$ -chain of relative molecular mass 35,000 ( $M_r$  35K) and a 45K partner (termed TCR  $\delta$ ). This TCR  $\gamma\delta$  is first detected approximately two days before the appearance of cell-surface TCR  $\alpha\beta$  heterodimers. We report that *N*-glycosidase digestions reveal that all of the  $\gamma$ -protein expressed on fetal thymocytes, as in adult CD4<sup>+</sup>8<sup>−</sup> (L3T4<sup>−</sup>, Lyt2<sup>−</sup>) thymocytes<sup>4</sup>, bear *N*-linked carbohydrate side chains. The major  $\gamma$ -gene transcribed in mature,  $\alpha\beta$ -bearing T cells ( $V_{\gamma 1.2}C_{\gamma 2}$ ) encodes no *N*-linked glycosylation site<sup>1</sup> so these results suggest that the fetal  $\gamma\delta$  receptor defines a distinct T-cell lineage

whose development in the thymus precedes classical  $\alpha\beta$ -bearing cells.

To characterize the surface CD3(T3)-associated receptor complex, immunoprecipitations were performed on surface radioiodinated fetal thymocytes of different gestational age with an anti-CD3 monoclonal antibody (mAb) (145-2C11)<sup>5,6</sup> and an anti-TCR/CD3  $\gamma$  antibody<sup>4</sup>. Figure 1 shows the results of immunoprecipitations with the anti-CD3 and anti- $\gamma$  on radioiodinated fetal thymocytes from gestational days 15, 17½ and 20 (newborn). Samples were electrophoresed on SDS-polyacrylamide gel electrophoresis (PAGE) gels under reducing conditions. On day-15 thymocytes, both the anti-CD3 and anti- $\gamma$  immunoprecipitated a 35K and a 45K protein. (The murine CD3 components on these cells do not label well using this lactoperoxidase procedure and are thus not easily visualized.) The  $M_r$ s of the two precipitated proteins are different from either TCR $\alpha$  or  $\beta$  which range from 37K to 43K as seen with an anti-CD3 precipitation of whole spleen (Fig. 1a, lane 5). On day 17½, the anti- $\gamma$  precipitation shows larger amounts of the same 35K and 45K proteins; however, in addition to these bands, anti-CD3 also precipitates significant amounts of 37–43K material corresponding to the  $\alpha\beta$  heterodimer. The identity of the 37–43K protein was confirmed by immunoprecipitation with an anti-TCR  $\beta$ -peptide serum (data not shown). By day 20, only the  $\alpha\beta$  heterodimer is seen in the anti-CD3 immunoprecipitate, although a small amount of the 35K and 45K proteins is present in the anti- $\gamma$  immunoprecipitate. These results indicate that the first CD3-associated receptor to arise in ontogeny (day 15) is a non- $\alpha\beta$  heterodimer containing the  $\gamma$ -chain and a second protein which we term TCR $\delta$  in accordance with the nomenclature suggested by Brenner *et al.*<sup>2</sup>. Both the  $\gamma\delta$  and  $\alpha\beta$  receptors are present in similar amounts by day 17½ at a time when most thymocytes are CD4<sup>+</sup>8<sup>+</sup> and 'mature' CD4<sup>+</sup>8<sup>−</sup> thymocytes have begun to appear<sup>7</sup>. At the time of birth (day 20) the  $\alpha\beta$  heterodimer is the predominant CD3-associated heterodimer.



**Fig. 1** Differential expression of two distinct T-cell receptors in thymocyte development. Unfractionated fetal thymocytes from day 15, 17½ and 20 (d15, d17.5, d20) of gestation, CD4<sup>+</sup>8<sup>+</sup> cells from day 17½, and adult spleen cells were surface radioiodinated and lysed in either digitonin or Nonidet-P-40 (NP-40). Lysates were then immunoprecipitated with *a*, anti-T3 or *b*, anti-γ-peptide antibody. Immunoprecipitated proteins were separated on SDS-PAGE gels using reducing conditions. Size markers, *M<sub>r</sub>*, in thousands.

**Methods.** Fetal thymocytes were prepared by mincing fetal thymuses from timed pregnancies. CD4<sup>+</sup>8<sup>+</sup> day-17½ fetal thymocytes were prepared by cytotoxic elimination with GK1.5 (rat anti-mouse L3T4) facilitated with MAR 18.5 (mouse anti-rat κ chain) and complement. Cells (1–5 × 10<sup>7</sup>) were <sup>125</sup>I-labelled using lactoperoxidase as previously described<sup>4</sup>. Cells were then lysed in 0.5% NP-40 (for anti-γ precipitations) or 1% digitonin buffer (for anti-T3 immunoprecipitations) containing phenylmethylsulphonyl fluoride, leupeptin, pepstatin and chymostatin. Samples were pre-cleared with rabbit anti-chicken immunoglobulin. Anti-CD3 immunoprecipitations were performed with culture supernatants from the 145-2C11 hybridoma and anti-γ immunoprecipitations were performed with rabbit anti-γ serum<sup>8</sup> affinity purified on a γ-peptide column as described<sup>15</sup>. Immune complexes were collected with protein A agarose beads and run under reducing conditions on 10% SDS-PAGE gels. Gels were dried and labelled and proteins were visualized using Kodak XAR-5 autoradiographic film at –70 °C with enhancing screens.

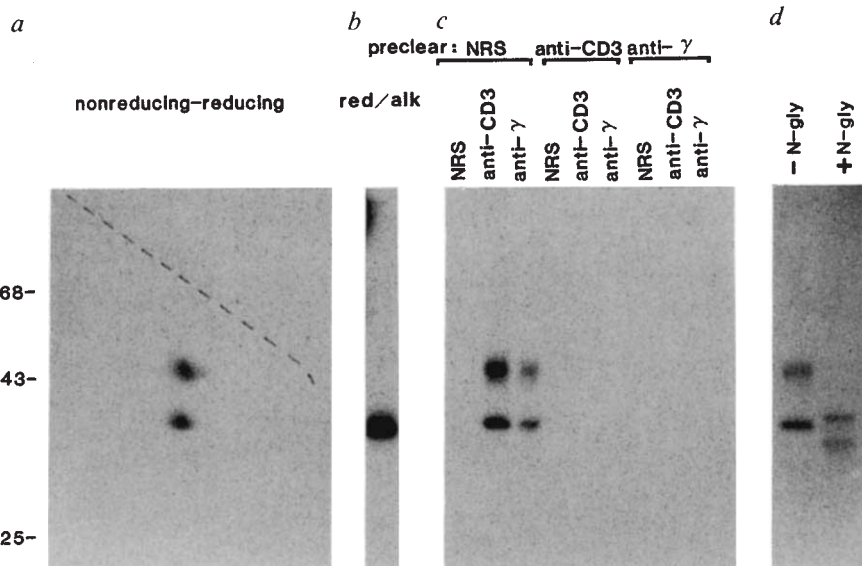
**Fig. 2** Biochemical characterization of the TCR γδ in fetal thymocytes. Day-15 fetal thymocytes were radioiodinated and lysed in digitonin as in Fig. 1 and the following biochemical analyses were performed. *a*, Anti-CD3 immunoprecipitates were run without reduction in the first dimension and then under reducing conditions in the second dimension. The diagonal, as determined by the positions of prestained, nondisulphide-linked standards, is shown with the dotted line. Size markers, *M<sub>r</sub>*, in thousands. *b*, Anti-CD3 immunoprecipitates were reduced with dithiothreitol, alkylated with iodoacetamide, reimmunoprecipitated with anti-γ and run on a 10% reducing SDS-PAGE gel. *c*, Lysates were precleared by immunoprecipitating with normal rabbit antiserum, anti-γ or anti-CD3. Each precleared sample was split three ways and immunoprecipitated with normal rabbit antiserum, anti-γ or anti-CD3. *d*, Anti-CD3 immunoprecipitates were divided. Half was treated with *N*-glycosidase-F (Genzyme) (lane 2) and half was not (lane 1). After treatment, samples were run on 10% reducing SDS-PAGE gels.

**Methods.** Cells were isolated, radioiodinated and immunoprecipitated as in Fig. 1. For diagonal gels (nonreducing–reducing), samples were eluted in nonreducing sample buffer and run for 12 h in a 10% SDS-PAGE tube gel followed by a 12.5% reducing slab gel. Reduction-alkylation, sequential immunoprecipitations and *N*-glycosidase-F treatment were performed as described<sup>4,16</sup>.

We were able to correlate TCR expression to surface phenotype by examining receptor expression on day-17½ fetal thymocytes depleted of CD4<sup>+</sup>8<sup>+</sup> (double positive) and CD4<sup>+</sup>8<sup>+</sup> (single positive) cells by cytotoxic elimination with GK1.5 (anti-CD4) mAb. As CD4<sup>+</sup>8<sup>+</sup> cells are not yet present at this gestational age<sup>7</sup>, the remaining cells were exclusively CD4<sup>+</sup>8<sup>+</sup> (data not shown). Immunoprecipitation of this subset with anti-CD3 showed significant amounts of the γδ receptor and little detectable αβ receptor (Fig. 1*a*, lane 4). This result indicates that during development, as in the adult thymus<sup>8</sup>, the γδ receptor is found within the double-negative subset whereas the αβ receptor is present predominantly on the single positive and probably double positive subsets.

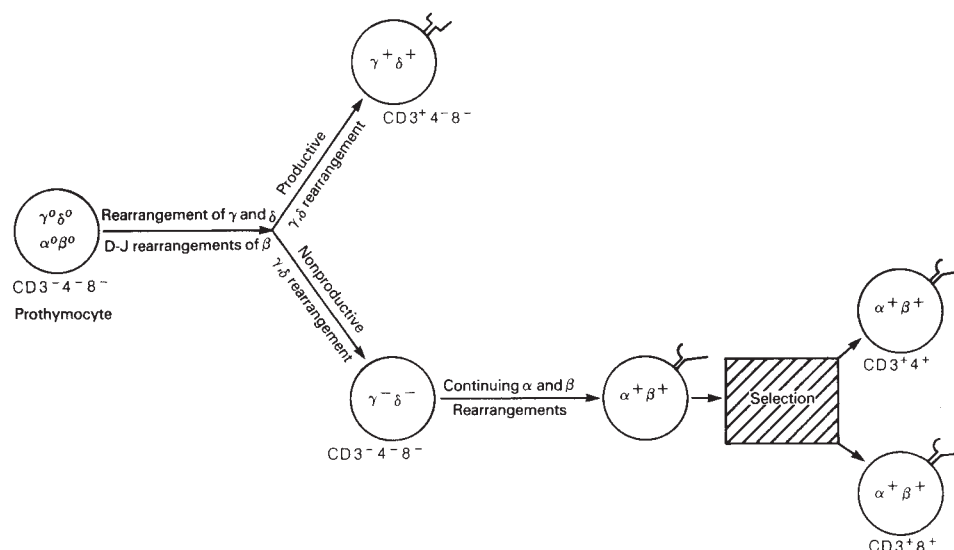
As with double-negative cells from the adult thymus<sup>4</sup>, the γ- and δ-chains in developing murine thymocytes are disulphide-linked. This was demonstrated by performing off-diagonal gels (first dimension under nonreduced conditions, second dimension under reduced conditions) on anti-CD3 precipitates of day-15 fetal thymocytes (Fig. 2*a*). Both the 35K and 45K proteins fall below the diagonal, indicating that they ran as a single disulphide-linked 80K molecule under nonreducing conditions.

To determine which of the two proteins reacted specifically with the anti-γ serum, anti-CD3 precipitates of day-15 thymocytes were reduced (with dithiothreitol) and alkylated (with iodoacetamide) to separate the γ- and δ-chains and then repre-





**Fig. 3** Model for developmental expression of two distinct T-cell receptors. The model incorporates previous data on TCR gene rearrangement<sup>8,10-12</sup> and our current results on receptor protein expression in developing thymocytes. Synchronous rearrangement of TCR  $\gamma$ - and  $\beta$ -genes is envisioned to occur in prothymocytes on entry into the thymus. Completed rearrangements occur in the  $\gamma$ - and  $\delta$ -genes at a point when rearrangements of the  $\beta$ -gene are partial. If productive  $\gamma$  and  $\delta$  rearrangements occur, a CD3- $\gamma\delta$  receptor is produced, halting further rearrangements of the  $\beta$ - and  $\alpha$ -gene, committing the cell to this lineage. If a nonproductive  $\gamma$  or  $\delta$  rearrangement occurs, the cell continues to rearrange its  $\alpha$ - and  $\beta$ -genes and can become a CD3- $\alpha\beta$  bearing cell subject to intrathymic selection.



cipitated with the anti- $\gamma$  serum (Fig. 2b). Under these conditions, only the 35K protein is seen, indicating that this is the  $\gamma$ -chain.

To demonstrate definitively the association of CD3 with the  $\gamma\delta$  heterodimer, sequential immunoprecipitations were performed on lysates from surface radioiodinated day-15 fetal thymocytes. Figure 2c shows that when these lysates were precleared with anti-CD3, all the anti- $\gamma$  reactive protein was removed. Similarly, when the lysates were precleared with the anti- $\gamma$  serum, virtually all the detectable CD3-associated receptor chains were removed. These results show that all the surface  $\gamma$ -chain is CD3-associated. Because all of the sequential immunoprecipitations were performed on lysates from freshly isolated cells, we conclude that the surface  $\gamma$ -chain is only present on the small subset of CD3-positive fetal thymocytes despite the fact that most express  $\gamma$ -specific mRNA<sup>6</sup>.

Analysis of the *N*-linked glycosylation pattern of the murine  $\gamma$ -chain can distinguish between expression of different  $\gamma$ -gene products. Because no  $C_{\gamma 4}$ -specific RNA is found in fetal thymocytes<sup>9</sup> and  $C_{\gamma 3}$  is a pseudogene<sup>10</sup>, the expressed  $\gamma$ -chain on fetal thymocytes could be only derived from  $C_{\gamma 1}$  or  $C_{\gamma 2}$  (using the nomenclature proposed by Garman *et al.*<sup>11</sup>).  $C_{\gamma 1}$  possesses one *N*-linked carbohydrate acceptor sequence<sup>11</sup>, whereas  $C_{\gamma 2}$  (and  $V_{\gamma 1.2}$  to which it rearranges) has no *N*-linked carbohydrate acceptor sites<sup>11,12</sup>. Figure 2d demonstrates that both the  $\gamma$ - and  $\delta$ -chains in fetal thymocytes are sensitive to *N*-glycosidase-F, an enzyme which cleaves *N*-linked carbohydrates. The  $\delta$ -chain reduces to a  $M_r$  of 37K and the  $\gamma$ -chain to 32K. The finding that all of the fetal thymic  $\gamma$ -chain is *N*-glycosylated indicates that it is all derived from  $C_{\gamma 1}$  rearrangements.

Our demonstration that the  $\gamma$ -chain in freshly isolated populations of developing fetal thymocytes is CD3-associated, disulphide-bonded to a 45K  $\delta$ -chain and primarily derived from  $C_{\gamma 1}$  rearrangements indicates that the cells which bear this receptor early in ontogeny are analogous to the CD3<sup>+</sup>4<sup>+</sup>8<sup>-</sup> cells in the adult thymus, whose  $\gamma$ -chain has recently been shown to have similar biochemical properties<sup>4</sup>. It is difficult to reconcile our

findings with models that directly link  $\gamma$ -chain expression on thymic precursors to MHC restriction of  $\alpha\beta$ -bearing T cells, either through developmental selection of  $\gamma\beta$  heterodimers<sup>8,13</sup> or reexpression of selected  $\gamma\delta$  receptors on mature T cells<sup>3,14</sup>. Either of these models predicts that the same  $\gamma$ -chain would be expressed in developing thymocytes as in mature  $\alpha\beta$ -bearing T cells (that is  $C_{\gamma 2}$ - $V_{\gamma 1.2}$ ). The predominant expression of the glycosylated  $\gamma$  species (derived for  $C_{\gamma 1}$ ) in both the fetal and adult thymus of B6 mice instead suggests that the  $\gamma\delta$  receptor may define a separate T-cell lineage whose intrathymic development precedes that of classic  $\alpha\beta$ -expressing T cells (Fig. 3). The presence of  $\gamma$ -gene rearrangements in mature cells with the  $\alpha\beta$  receptor<sup>12</sup> as well as partial  $\beta$ -gene rearrangements in cells that bear the  $\gamma\delta$  receptor<sup>4</sup> indicates that both lineages may derive from a common precursor. Recent evidence suggests that immature thymocytes begin to rearrange their TCR  $\gamma$  and  $\beta$  genes synchronously<sup>10</sup>. However, the  $\gamma$  (and possibly  $\delta$ ) locus completes a *V-J-C* rearrangement in most thymocytes by day 15 at a time when many  $\beta$ -locus rearrangements are incomplete (*D-J-C*)<sup>10</sup>. Therefore, as indicated here, the  $\gamma\delta$  heterodimer is the first T-cell receptor to appear in ontogeny. As thymocytes with the  $\gamma\delta$  heterodimer predominantly express 1.0-kilobase-gene messenger RNA<sup>4</sup>, which is the product of an incomplete *D-J* rearrangement, it is likely that once the product of a productively rearranged  $\gamma$ - and  $\delta$ -gene is synthesized, further rearrangement of  $\beta$ - (and  $\alpha$ -) genes does not occur. If the rearrangement of  $\gamma$  or  $\delta$  is nonproductive, no full length protein is produced and the cell continues to rearrange its  $\beta$ - and  $\alpha$ -genes in an attempt to produce an  $\alpha\beta$  heterodimer. This model is compatible with the presence of full-length (1.3-kb)  $\beta$  mRNA in CD3<sup>+</sup>4<sup>+</sup>8<sup>-</sup> cells<sup>4,8</sup> and predicts a high frequency of nonproductive  $C_{\gamma 1}$  rearrangements in mature  $\alpha\beta$ -bearing T cells.

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# Characterization of murine thymocytes with CD3-associated T-cell receptor structures

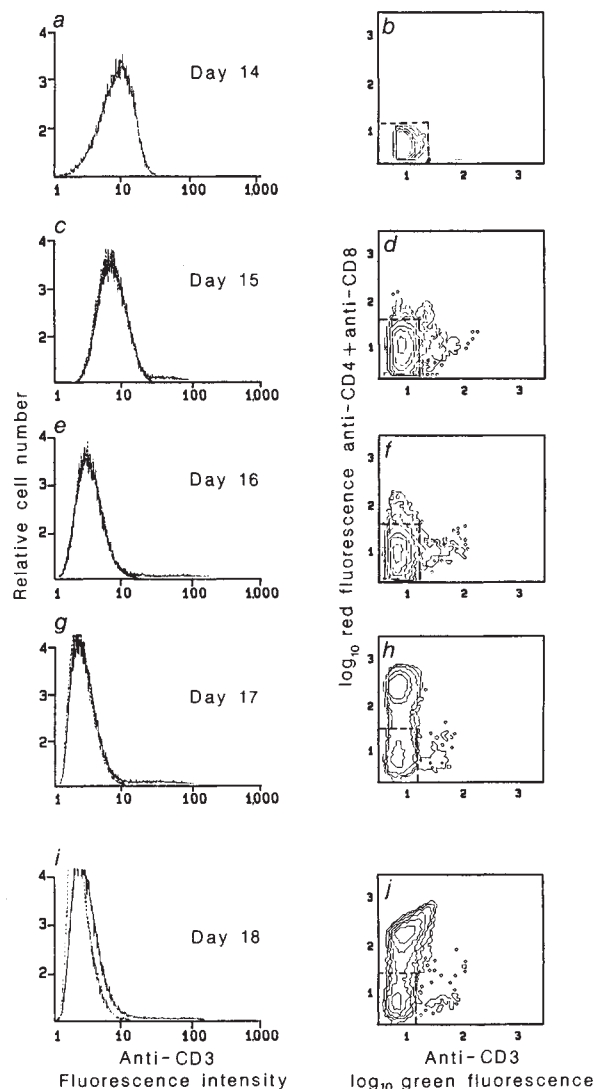
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The thymus is the major site for T-cell receptor (TCR) gene rearrangement and T-cell maturation<sup>1,2</sup>. The specific antigen recognition structure (TCR) on murine T cells has been shown to be dependent on a polymorphic set of disulphide-linked heterodimers, containing two integral membrane glycoprotein chains, TCR $\alpha$  and TCR $\beta$ , expressed in non-covalent association with an invariant complex of proteins, CD3 (T3)<sup>3-6</sup>. Recently, a novel TCR/CD3 complex, that includes the product of the TCR $\gamma$  gene, has been identified on a subset of both peripheral cells and thymocytes<sup>7-9</sup>. Here we examine the expression of TCR/CD3 complexes in fetal ontogeny and in the adult thymus. The results

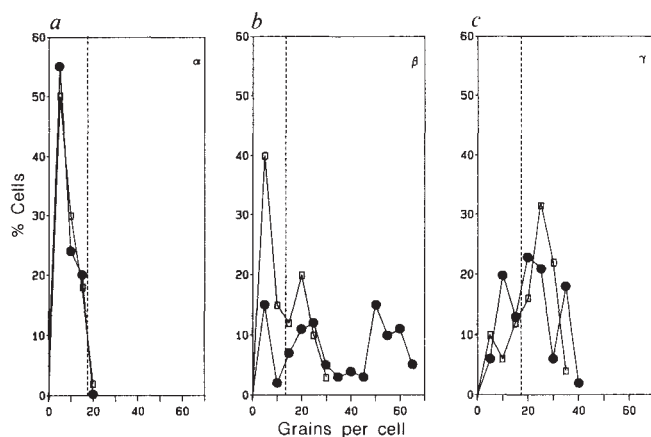
demonstrate that CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup>(T3<sup>+</sup>, L3T4<sup>-</sup>, Lyt2<sup>-</sup>) cells are detected in day-15 fetal thymi, throughout fetal development and in adult thymus. *In situ* hybridization studies indicate that these early CD3<sup>+</sup> cells express high levels of TCR $\gamma$ -specific RNA, low levels of TCR $\beta$ -specific RNA and no detectable TCR $\alpha$ -specific RNA. Day-16 CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup> fetal thymocytes can be activated to proliferate and demonstrate cytolytic activity when cultured in the presence of anti-CD3 monoclonal antibodies and interleukin-2 (IL-2). These results suggest that CD3-bearing cells, present early in thymic ontogeny, express a functional TCR and may, therefore, be important in repertoire development.

The availability of a newly derived anti-murine CD3 monoclonal antibody (mAb) specific for the relative molecular mass 25,000 (*M*, 25 K) protein component of the TCR complex (designated CD3- $\epsilon$ )<sup>10</sup> has provided a means to study the developing fetal thymus for the presence of CD3<sup>+</sup> thymocytes. Flow cytometric (FCM) analysis demonstrated that a subpopulation of CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup> fetal thymocytes were first evident on day 15 of gestation (Fig. 1*c-d*) and remained present throughout thymic development (Fig. 1*f, h, j*). On day 18 two additional populations of CD3<sup>+</sup> cells were evident. A significant percentage of the CD4<sup>+</sup>8<sup>+</sup> cells were CD3 dull (Fig. 1*i, j*) and in some experiments a small, but significant percentage of CD3 bright, CD4<sup>+</sup>8<sup>-</sup> cells was observed (data not shown). The early CD3<sup>+</sup> fetal thymocytes were analysed for TCR $\alpha$ ,  $\beta$ , and  $\gamma$ -specific RNA expression by *in situ* hybridization which requires very few cells, examines RNA at the single cell level, and thus shows the population distribution of gene expression. Day-16 fetal



**Fig. 1** Multicolour flow cytometry (FCM) analysis of cell-surface CD3 expression in fetal thymus ontogeny. Thymocytes from day-14 (*a, b*), day-15 (*c, d*), day-16 (*e, f*), day-17 (*g, h*) or day-18 (*i, j*) embryos were reacted with the anti-CD3 mAb<sup>20</sup> (solid line, *a, c, e, g, i*) or a control negative hamster mAb (dotted line, *a, c, e, g, i*) followed by FITC-conjugated mouse anti-hamster-immunoglobulin antibody (FITC-M $\alpha$ H). Fetal thymocytes were also reacted with anti-CD3 mAb followed by FITC-M $\alpha$ H followed by biotin-conjugated anti-CD8 (Becton-Dickinson) plus biotin-conjugated anti-CD4 (ref. 21) followed by Texas-Red streptavidin (Bethesda Research) (*b, d, f, h, j*). Immunofluorescence staining and washing were performed as described<sup>10</sup> using aliquots of  $1 \times 10^6$  cells per tube. All procedures were performed in Hanks balanced salt solution containing 0.1% bovine serum albumin and 0.1% sodium azide. FCM analysis was performed as described<sup>21</sup> using a B-D Dual Laser FACS II equipped with the manufacturer's filters and photomultiplier tubes, an argon ion laser for FITC excitation (488 nm) and a pumped dye (rhodamine 6G) laser for Texas-Red excitation (590 nm) and interfaced to a Digital Equipment Corporation PDP 11/24 computer. All analyses were performed using logarithmic (three decades) amplification and data were collected and analysed using hardware and software designed by the Computer Systems Laboratory of the Division of Computer Research and Technology at the National Institutes of Health. Fluorescence data were collected on 50,000 viable cells as determined by forward light scatter and propidium iodide gating. One colour (FITC) data were displayed as increasing fluorescence intensity (*x*-axis) versus cell number (*y*-axis) (*a, c, e, g, i*). Two-colour data were displayed as contour diagrams (*b, d, f, h, j*), with logarithmically increasing intensities of green (FITC) fluorescence are plotted on the *x*-axis versus logarithmically increasing intensities of red (Texas-Red) fluorescence on the *y*-axis. Constant values of the percentage of total cell number on the *z*-axis were selected to draw the rings or contours around peaks of cells correlating green and red fluorescence. Cells defined to be negative for both red and green fluorescence are shown by boxes in the lower left portion of the contour diagrams. The *x* and *y* coordinates which defined negative cells were selected as the intersection of positive and control negative profiles in each parameter.





**Fig. 2** *In situ* hybridization of RNA in day-16 CD3<sup>+</sup> (□) and CD3<sup>-</sup> (●) fetal thymocytes. Day-16 fetal thymocytes were reacted with anti-CD3 mAb followed by FITC-MαH. CD3 positive and negative cells were determined by FCM analysis and separated by electronic cell sorting using a Becton-Dickinson FACS II. The CD3<sup>+</sup> cells were >97% pure and the CD3<sup>-</sup> cells were >99% pure as reanalysed by FCM. Sorted thymocytes were cytospun onto glass slides and fixed with paraformaldehyde in phosphate buffered saline (PBS) and 70% ethanol. *In situ* hybridization was performed with *a*, TCR Cα; *b*, TCR Cβ and *c*, TCR V<sub>γ1.2</sub>C<sub>γ2</sub>-specific <sup>35</sup>S-labelled RNA probes using a modification of a previously described protocol<sup>22</sup>. Gene inserts were cloned into pGEM1 vectors (Promega Biotec), linearized and transcription was performed with either T7 or SP6 RNA polymerase. Hybridization was performed at 50 °C for 12 h at a concentration of 1 × 10<sup>8</sup> c.p.m. ml<sup>-1</sup>. Slides were washed at 54 °C in 2 × SSC/50% formamide, followed by incubation with 100 μg ml<sup>-1</sup> RNase A and 1 μg ml<sup>-1</sup> RNase T<sub>1</sub> for 30 min at 37 °C. Slides were dried and autoradiography was performed with NTB2 photographic emulsion (Kodak). Exposure times were 10 days for TCRα, 5 days for TCRβ and 20 days for TCRγ. Grains were counted on 50–150 cells per slide. Dashed lines represent the maximal number of grains observed in the negative control (RNA sense strand probes). Therefore, points to the right of the dashed lines are considered positive by the limits of this technique.

thymocytes were stained with the anti-CD3 mAb and separated into CD3<sup>+</sup> and CD3<sup>-</sup> subsets by electronic cell sorting. The sorted cells were analysed for TCRα, β, and γ-specific RNA as described elsewhere (D. Pardoll *et al.*, in preparation). As seen in Fig. 2a, no significant amount of TCRα RNA was observed in either the CD3<sup>+</sup> or CD3<sup>-</sup> subpopulations. In contrast, TCRγ RNA was present in most of the CD3<sup>+</sup> and CD3<sup>-</sup> thymocytes (Fig. 2c). Examination of TCRβ RNA showed that among the CD3<sup>-</sup> fetal thymocytes ~50% expressed high levels (>40 grains per cell) and ~35% of the cells expressed low levels of TCRβ chain-specific RNA. In contrast, 55% of the CD3<sup>+</sup> cells did not express detectable levels of TCRβ RNA and the remainder expressed only low levels (<40 grains per cell) that may represent TCRβ genes that have undergone a *D-J* rearrangement as seen in adult CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup> thymocytes<sup>6</sup>. Thus, the majority of the CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup> thymocytes express TCRγ RNA but low or undetectable amounts of mature TCRβ and no TCRα-specific RNA. In the accompanying study<sup>11</sup>, these CD3<sup>+</sup> cells were found to express a second T cell receptor (TCRγδ) associated with the CD3 complex.

CD3<sup>+</sup> cells present early in fetal ontogeny were examined for functional activity using the anti-CD3 mAb and the anti-Vβ8 (F23.1) mAb as described<sup>10</sup>. Day-16 fetal thymocytes were harvested and incubated for 5 days with the anti-murine CD3 mAb in the presence or absence of co-stimulating factors. As indicated in Table 1, the anti-CD3 antibody induced significant proliferation of mature peripheral T cells in the absence of exogenous factors. Adult and fetal thymocyte proliferation, however,

**Table 1** Activation of fetal and adult thymocytes by anti-CD3 mAb

**a. Proliferation**

| Responder*       | IL-2† | T cell proliferation (c.p.m. × 10 <sup>-3</sup> ) |                     |                  |
|------------------|-------|---------------------------------------------------|---------------------|------------------|
|                  |       | medium                                            | 145-2C11 (Anti-CD3) | F23.1 (Anti-Vβ8) |
| B10 Spleen       | —     | 0.4                                               | 86.4                | 1.3              |
| B10 Spleen       | +     | 5.5                                               | 164.7               | 79.8             |
| B10 Fetal thymus | —     | 0.8                                               | 0.7                 | 0.8              |
| (day 14)         | +     | 3.7                                               | 6.9                 | 3.3              |
| B10 Fetal thymus | —     | 0.7                                               | 2.6                 | 1.1              |
| (day 16)         | +     | 3.9                                               | 68.4                | 3.1              |
| B10 Adult thymus | —     | 0.3                                               | 1.9                 | 0.5              |
| B10 Adult thymus | +     | 4.1                                               | 13.8                | 9.3              |

**b. Cytolysis**

| Effectors‡                | Cultured with   | mAb added | % Specific lysis§ |
|---------------------------|-----------------|-----------|-------------------|
| B10 Adult spleen          | —               | —         | —4.2%             |
|                           | —               | Anti-CD3  | 28.2%             |
|                           | Anti-CD3        | —         | 37.9%             |
| B10 Fetal thymus (day 16) | —               | Anti-CD3  | 74.2%             |
|                           | —               | —         | 1.1%              |
|                           | —               | Anti-CD3  | 6.8%              |
|                           | Anti-CD3 + IL-2 | —         | 25.6%             |
|                           | Anti-CD3 + IL-2 | Anti-CD3  | 58.8%             |

\* 1 × 10<sup>5</sup> cells were cultured in culture media<sup>10</sup> for 96 h in round-bottom microtitre wells in 7.5% CO<sub>2</sub> at 37 °C. The <sup>3</sup>H-thymidine (1 μCi) was added to each during the last 16 h of incubation. Radiolabel incorporation was determined as described<sup>10</sup>.

† Human recombinant IL-2, 50 units ml<sup>-1</sup> (Cetus).

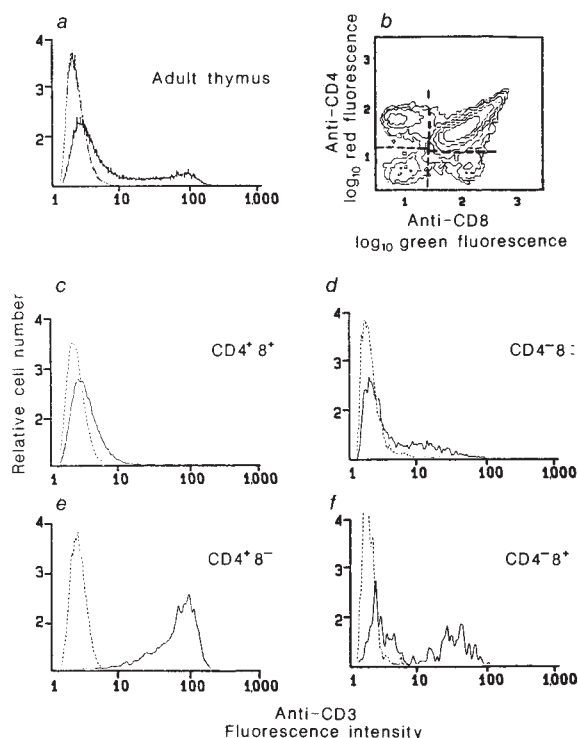
‡ 1 × 10<sup>6</sup> cells were cultured for 7 days as above in the presence or absence of anti-CD3 (50 μl of a high-titred culture supernatant) and IL-2. No activation was observed in the presence of anti-CD3 or IL-2 alone (data not shown).

§ Lysis was assessed on FcR-bearing LPS blast cells as previously described<sup>10</sup>; effector: target ratio, 20:1.

required exogenous recombinant IL-2. These findings suggest that the requirements for the activation of fetal and adult thymocytes by anti-CD3 mAb may be different than those for mature peripheral T cells. This could be due to differences in the differentiation states of the two populations or could be a result of the presence or absence of another cell population critical for activation. Anti-Vβ8-specific mAb (F23.1) induced the proliferation of adult thymus and peripheral T cells but not day-16 fetal thymocytes, consistent with the absence of F23.1-bearing cells before day 17 of fetal development<sup>13</sup>.

Anti-CD3 activated fetal thymocytes were also analysed for cytolytic activity. The anti-CD3 mAb has previously been shown to effectively redirect lysis by murine alloreactive CTL (cytotoxic T lymphocytes) to Fc receptor (FcR)-bearing target cells that do not bear the nominal antigen<sup>10</sup>. As shown in Table 1b, anti-CD3 plus IL-2-activated fetal thymocytes reacted with syngeneic lipopolysaccharide (LPS)-induced FcR<sup>+</sup> B-cell blasts. The efficient lysis of the LPS blasts depended on the presence of anti-CD3 antibody during the culture period and in the <sup>51</sup>Cr-release assay. In several studies lysis of natural killer (NK)-sensitive YAC tumour cells was also observed. This killing was not completely inhibited by anti-FcR antibodies suggesting that anti-CD3-induced cytolytic cells had some NK-like activity. Thus, it would appear that anti-CD3 antibodies induced proliferation and cytolytic activity of CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup> fetal thymocytes. Finally, the activated T cells were examined for expression of different differentiation antigens. FCM analysis of cultured day-16 fetal thymocytes showed that the phenotype of the proliferating cells at the time of cytolytic assay was CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup> and Vβ8<sup>-</sup> consistent with the conclusion that the cytolytic cells are a CD3<sup>+</sup>, αβ<sup>-</sup> T-cell population.

The presence of CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup> cells early in fetal ontogeny suggested a possible role for these cells in thymus development



**Fig. 3** Three-colour FCM analysis of adult thymocytes. *a*, CD3 expression on whole thymocytes; *b*, CD4 versus CD8 expression on whole adult thymocytes; *c-f*, thymocyte subpopulations as determined by software gating: *c*,  $CD4^+8^+$ ; *d*,  $CD4^-8^-$ ; *e*,  $CD4^+8^-$ ; *f*,  $CD4^-8^+$  cells. Solid line, anti-CD3; dotted line, irrelevant hamster mAb followed by FITC-labelled M $\alpha$ H single-parameter green fluorescence profiles (*a, c, d, e, f*). After anti-CD3 or control mAb plus FITC-M $\alpha$ H, cells were reacted with biotin-conjugated anti-CD8 and phycoerythrin (PE)-conjugated anti-CD4 (both Becton-Dickinson) followed by Texas-Red streptavidin. List mode data (light scatter, FITC fluorescence, PE fluorescence and Texas-Red fluorescence) were collected on 50,000 viable cells as determined by forward light scatter intensity. *b*, Representative contour diagram of anti-CD8 versus anti-CD4 fluorescence. A similar profile was used to select coordinates for software gating on thymocyte subsets defined by CD8 and CD4. Results of software gating and resulting FITC profiles are shown in panels *c-f*.

during ontogeny and adult life. Therefore, CD3 expression was assessed on the distinct adult thymus subpopulations that have been shown to include dull- $CD5^+$  ( $Ly-1^+$ ),  $CD4^-8^-$  thymocytes capable of differentiating into cortical-type thymocytes *in vitro* and both cortical and mature medullary-type T cells *in vivo*<sup>14,15</sup>,  $CD4^+8^+$  cortical-type thymocytes that for the most part die within the thymus, and  $CD4^+8^-$  and  $CD4^-8^+$  mature, medullary thymocytes<sup>15,16</sup>. First, single-colour FCM was performed to assess the expression of CD3 on adult thymocyte populations (Fig. 3). Two distinct  $CD3^+$  populations were observed (Fig. 3*a*), a bright  $CD3^+$  subset of cells representing 10–15% of total thymocytes and a dull  $CD3^+$  subset representing at least 50% of the remaining thymocytes. To discriminate between the thymocyte subsets expressing the CD3 antigen further, adult thymocytes were analysed for coordinate expression of CD8, CD4, and CD3 by three-colour FCM. Figure 3*b* shows that analysis of CD4 and CD8 expression subdivides the thymocytes into four subpopulations ( $CD4^-8^-$ ;  $CD4^+8^+$ ;  $CD4^+8^-$ ;  $CD4^-8^+$ ). As in the fetal ontogeny studies shown above (Fig. 1) and in studies of human adult thymus<sup>17,18</sup>, a significant percentage of the  $CD4^-8^-$  cells expressed CD3 (Fig. 3*d*). The dull- $CD5^+$ ,  $CD4^-8^-$  subset of these cells have been previously shown to express TCR $\gamma\delta$ /CD3 on their cell surface<sup>7,9,11</sup>. The majority of bright- $CD5^+$ ,  $CD4^-8^-$  cells are also  $CD3^+$  (J.A.B. and B.J.F., unpublished observation). The TCR genes, expressed by this adult thymocyte population are at present under study. The major thymocyte population bears both CD4 and CD8 antigen and expresses low levels of CD3 (Fig. 3*c*) consistent with the conclusion that at least a portion of the cortical-type thymocytes express TCR/CD3 protein on their cell surface. But the level of CD3 expression was extremely low, suggesting that either very little TCR is expressed on these cells or the cell surface configuration of the TCR/CD3 complex alters the binding of the mAb.

The mature  $CD4^+8^-$  cells express the highest levels of CD3 (Fig. 3*e*) similar to the levels of CD3 seen on peripheral  $CD4^+8^-$  T cells (J.A.B. *et al.*, unpublished observations). Staining of the  $CD4^+8^-$  T cells with the anti-CD3 antibody resulted in the discrimination of two populations, one CD3-bright, probably the mature T cells, and one CD3-dull population that could be a precursor of the dull  $CD3^+4^+8^+$  cortical thymocytes as

hypothesized by Ceredig *et al.*<sup>19</sup> (Fig. 3*f*). CD3 expression was significantly higher on the mature  $CD4^+8^-$  cell population than on the  $CD4^-8^+$  cell population (median fluorescence of  $CD4^+8^- = 92.4$  mV; median fluorescence of  $CD4^-8^+ = 170.7$  mV), suggesting that different T-cell subsets express different levels of TCR/CD3 complex.

In summary, the data presented here and in the accompanying study<sup>11</sup> have shown that the CD3- $\epsilon$  molecule is detectable on a subpopulation of TCR $\gamma\delta^+$ ,  $CD4^-8^-$  thymocytes early in fetal development and in adult thymus. The function of these cells and the ligand for the TCR $\gamma\delta$ /CD3 receptor remains unclear, but we have shown that anti-CD3 mAb can activate these cells to proliferate, secrete lymphokines (data not shown) and develop cytolytic activity in culture.

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## Characterization of an expressed CD3-associated Ti $\gamma$ -chain reveals $C_\gamma$ domain polymorphism

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The majority of human T cells express an antigen receptor consisting of a disulphide-linked heterodimer (Ti) of relative molecular mass 80,000–90,000 ( $M_r$  80–90K) which is noncovalently associated with a set of at least three proteins of  $M_r$  20–28K termed CD3 (Leu4, T3)<sup>1–3</sup>. Whereas both chains of Ti, an acidic  $\alpha$ -chain of  $M_r$  48–54K and a more basic  $\beta$ -chain of  $M_r$  40–44K, contain variable and constant region domains, the component peptides of CD3 are invariant<sup>4–8</sup>. Several laboratories have more recently reported the expression of CD3 in association with a novel protein<sup>9–12</sup>. On the surface of long-term T-cell lines and one thymocyte clone this novel structure consists of a 40K protein noncovalently linked to a 55 or 62K protein<sup>9,10</sup> identified as the protein product of the Ti  $\gamma$ -chain gene, a T-cell specific gene which like the Ti  $\alpha$ - and Ti  $\beta$ -chain genes undergoes rearrangement of variable (V) and joining (J) region gene segments<sup>13–16</sup>. On the human T-cell leukaemic line PEER we have detected only a single 55K glycoprotein associated with CD3<sup>11</sup>. We here demonstrate that an anti-Ti  $\gamma$ -peptide antiserum reacts with the 55K CD3-associated

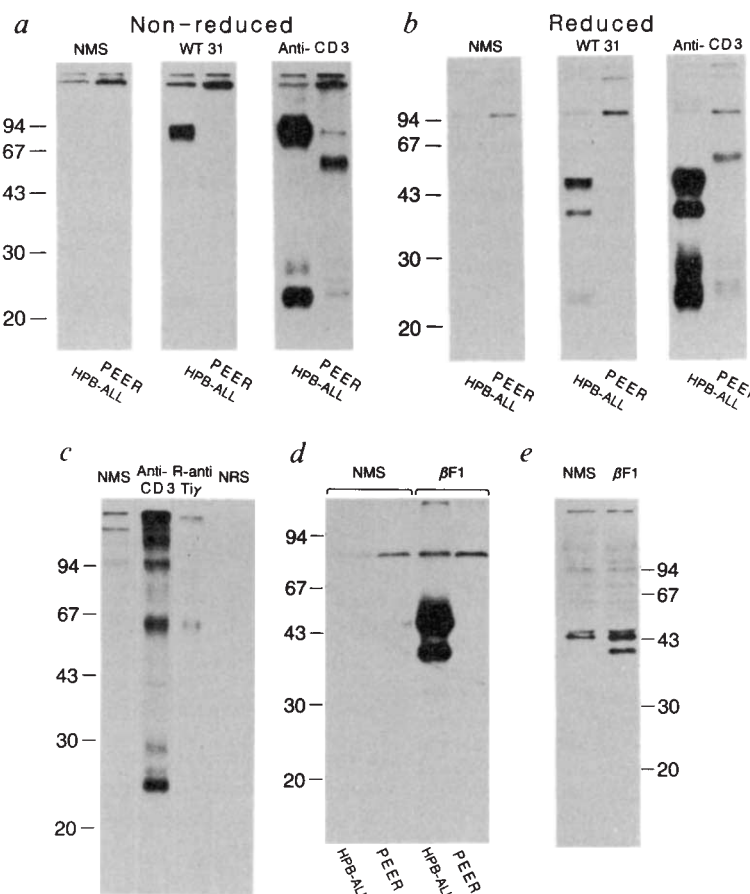
protein on PEER. Most previously described human Ti  $\gamma$ -chain complementary DNA clones encode the products of non-functional rearrangements<sup>17,18</sup>. One of the Ti  $\gamma$  cDNAs isolated from PEER, however, represents a functional rearrangement reported for the first time in a cell which expresses a Ti  $\gamma$ -chain protein product on the cell surface. Interestingly, a 48-base-pair (bp) sequence in the constant (C) region domain of this functional Ti  $\gamma$ -chain cDNA is triplicated in PEER and duplicated in other cDNAs isolated from PEER and other cell lines.

In previous studies, a single non-disulphide linked 55K protein was co-immunoprecipitated with CD3 isolated from iodinated cell-surface proteins of PEER<sup>11</sup>. This suggested that the CD3 antigen was not associated with a Ti  $\alpha/\beta$  heterodimer on PEER. To confirm this, we used the monoclonal antibody WT31, which reacts with nonpolymorphic determinants on Ti, and found that PEER failed to react with this antibody by immunoprecipitation (Fig. 1a and b) or by immunofluorescence<sup>11</sup>. In contrast, WT31 could be used to immunoprecipitate the typical Ti  $\alpha/\beta$  heterodimer expressed on the CD3-bearing leukaemic line, HPB-ALL.

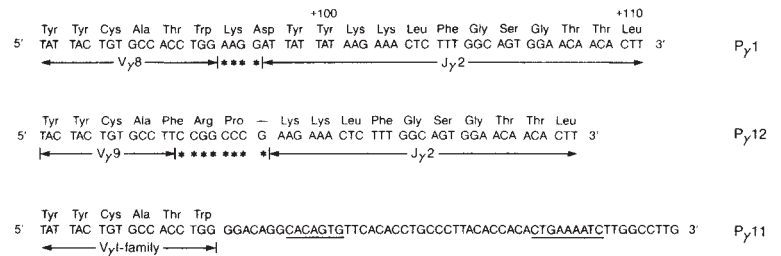
The CD3-associated 55K protein, which was noncovalently linked to a 40K protein on the surface of polyclonal T-cell lines, reacted with antisera generated against peptides derived from the predicted primary sequence of the Ti  $\gamma$ -chain<sup>9,10</sup>. Only one 55K chain appeared to be associated with CD3 on PEER, so it was of interest to determine whether this also represented a Ti  $\gamma$ -chain gene product. We found that one of these previously described anti-Ti- $\gamma$ -peptide heterosera, generated against a deduced constant region sequence, was reactive with the 55K protein on PEER (Fig. 1c). This finding supports the notion that this 55K protein is a product of the Ti  $\gamma$ -chain gene.

Ti  $\beta$ -chain expression precedes Ti  $\alpha$ -chain expression but may follow Ti  $\gamma$ -chain expression during thymocyte ontogeny<sup>19–21</sup>, so that the formation of Ti  $\gamma/\beta$ -chain heterodimers has been proposed<sup>19,22</sup>. PEER expresses both Ti  $\beta$ - and Ti  $\gamma$ -chain tran-

**Fig. 1** Characterization of the 55K CD3-associated glycoprotein expressed on PEER. **a**, **b**, WT31 fails to react with the 55K CD3-associated protein. Immunoprecipitation of cell surface radio-iodinated proteins solubilized from HPB-ALL or PEER in 5 mM CHAPS was performed with the indicated preformed immune complexes prepared with rabbit anti-mouse IgG and either normal mouse serum (NMS), WT31 ascites, or anti-CD3 (anti-Leu4) as described<sup>7,8</sup>. Electrophoresis of isolated precipitates was performed under non-reducing (**a**) or reducing (**b**) conditions in 11% sodium dodecyl sulphate (SDS) polyacrylamide gels. **c**, The 55K protein is recognized by an anti-Ti  $\gamma$  peptide serum. An anti-CD3 precipitate prepared from radio-iodinated PEER cells was denatured in 1% SDS containing 2 mM dithiothreitol and heated at 56 °C for 30 min. The sample was renatured with 4 vols of 1.5% Nonidet P40 (NP40), 20 mM iodoacetamide, in Tris-buffered saline, pH 7.4. Immunoprecipitation with normal rabbit serum (NRS) or rabbit anti-Ti- $\gamma$ -peptide (R-anti-Ti- $\gamma$ ) was performed according to the methods of Brenner *et al.*<sup>9</sup>. The rabbit anti-Ti- $\gamma$ -peptide serum was generated against a synthetic peptide of sequences 117–136 of ref. 18. Immunoprecipitates were analysed as above on 11% gels. **d**, **e**, The  $\beta$ -chain protein is not detected on the surface of PEER but is present intracellularly. Immunoprecipitates prepared from HPB-ALL and PEER cells which were radio-iodinated and lysed in 1% NP40 were isolated with NMS of  $\beta$ F1 monoclonal antibody and analysed as above. In **e**, PEER cell lysates were prepared from cells cultured in <sup>35</sup>S-methionine and <sup>35</sup>S-cysteine in 1% NP40 as described<sup>8</sup>. Immunoprecipitates isolated with either preformed complexes containing NMS or  $\beta$ F1 were analysed under reducing conditions in 11% SDS-polyacrylamide gels. Relative mobilities of standards with the indicated molecular masses are shown in the margins.

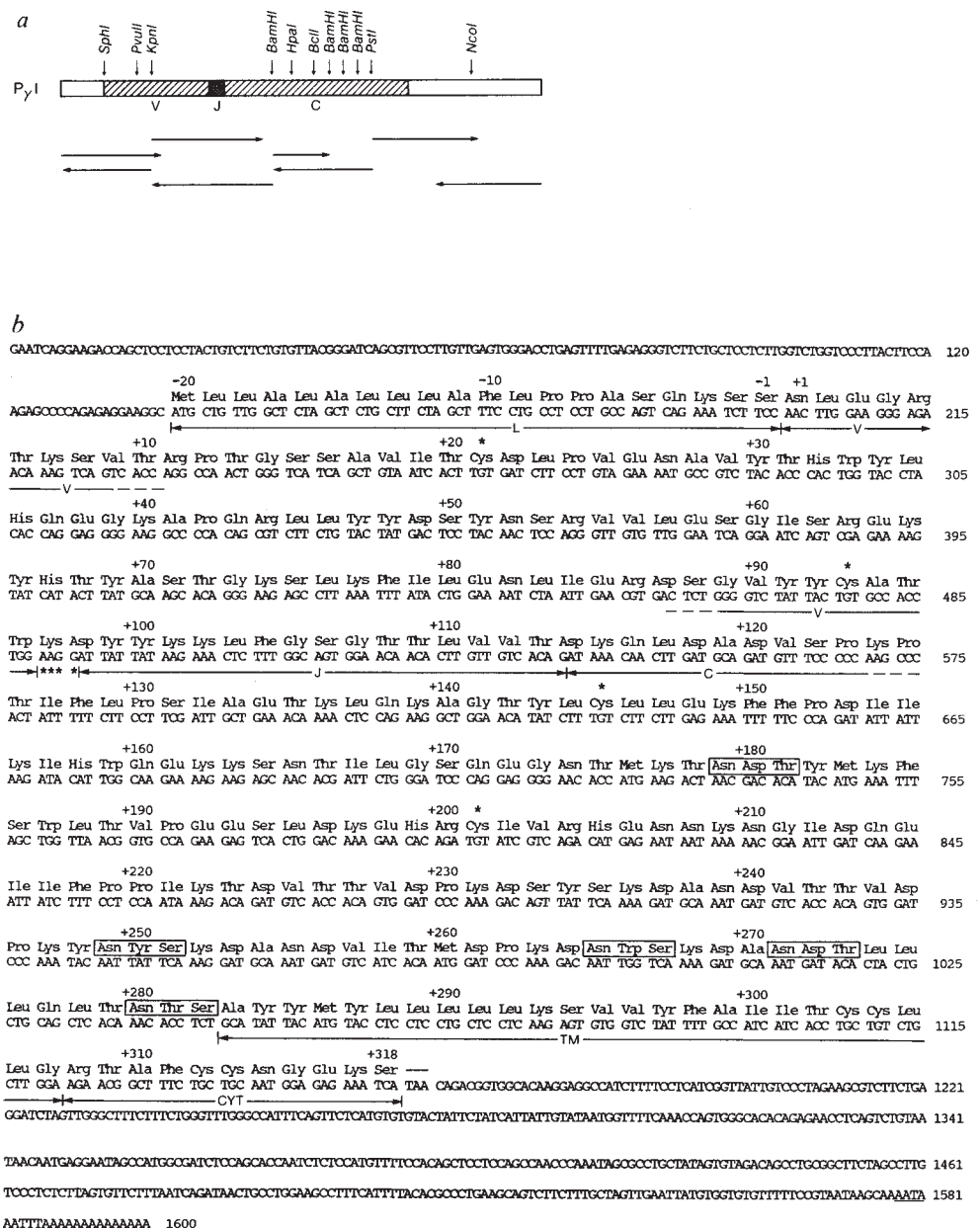


**Fig. 2** Nucleotide and predicted amino-acid sequence at the 3' end of the variable regions of the cDNA clones isolated from PEER. In-frame *V-J* rearrangement, with four additional junctional nucleotides (marked by asterisks) is found in *P<sub>γ</sub>1*, in which the *V<sub>γ</sub>8* gene, a member of the *V<sub>γ</sub>I* variable region subgroup, is rearranged to *J<sub>γ</sub>2*. The non-productive rearrangement on the other chromosome is represented by *P<sub>γ</sub>12*, in which the *V<sub>γ</sub>9* gene, a member of the *V<sub>γ</sub>II* subgroup, is rearranged to *J<sub>γ</sub>2*; there are eight nucleotides of unknown origin at the junction, and there is a deletion of several nucleotides from the *J* segment. The *P<sub>γ</sub>11* cDNA clone represents a 2.2-kilobase (kb) transcript of an unrearranged and previously undescribed pseudogene in the *V<sub>γ</sub>I* subgroup; the consensus heptamer and nanomer recombination signals, separated by 23 nucleotides, are underlined. *V* regions designated as in ref. 16.

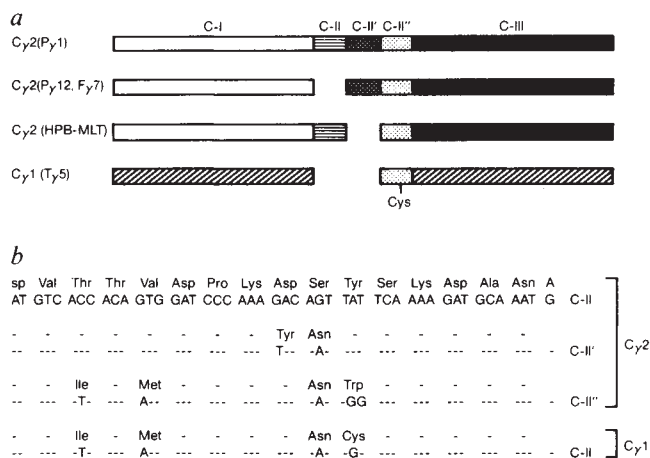


**Fig. 3** Nucleotide sequence of the *P<sub>γ</sub>1* cDNA clone and amino-acid sequence of the translated Ti  $\gamma$ -chain protein. *a*, Restriction map of the 1.6-kb *P<sub>γ</sub>1* cDNA clone; arrows, M13 inserts sequenced by the dideoxy chain termination procedure<sup>28</sup>. *b*, Sequence of the cDNA and the predicted protein. Numbers above the amino-acid sequence, residue positions in the mature protein; numbers on the right, nucleotide positions. Extracellular cysteines in the variable (*V*) and constant (*C*) regions are marked by stars. The leader sequence (*L*), *V* domain, *J* segment, and *C* region, which includes the transmembrane (*TM*) and cytoplasmic (*CYT*) domains, are indicated by horizontal arrows; \*, four nucleotides of uncertain origins at the *V*-*J* junction. The five potential sites for *N*-linked glycosylation (Asn-X-Thr or Asn-X-Ser) are boxed. The polyadenylation signal, AATAAA, is underlined.

**Methods.** A cDNA library was prepared in the vector  $\lambda$ gt10 using polyadenylated RNA from the T-cell leukaemic line, PEER. The procedure was as described<sup>29</sup>, except that size-selected cDNA larger than 1.0 kb was inserted into the *Eco*RI site of  $\lambda$ gt10. The library was screened with a full-length  $\gamma$  cDNA clone, F $\gamma$ 7, isolated from the T-cell leukaemia FRO2.2<sup>29</sup> (D. Littman, unpublished). Hybridization was performed at 42 °C in 50% formamide, 5 $\times$ SSCPE, 10% dextran sulphate, 1 $\times$ Denhardt's, and 100  $\mu$ g ml<sup>-1</sup> denatured salmon sperm DNA; the filters were washed in 2 $\times$ SSC, 0.1% SDS at 68 °C. Approximately 0.05% of plaques hybridized with the probe F $\gamma$ 7.







**Fig. 4** Alternative use of three C-II domains in transcripts of the  $C_{\gamma 2}$  locus. **a**, Schematic representation of exons in four  $C_{\gamma 2}$  cDNAs and one  $C_{\gamma 1}$  cDNA. F $\gamma 7$  is a cDNA clone isolated from a cDNA library of the FRO2.2 leukaemia (P.-G. Pelicci *et al.*, in preparation); T $\gamma 5$  is a cDNA clone isolated from a human peripheral T-cell library (D.L., unpublished result). The C-II domain of the  $C_{\gamma 1}$  locus differs from the C-II domain of  $C_{\gamma 2}$  at a single nucleotide, resulting in replacement of the tryptophan by a cysteine. **b**, Sequence of the three C-II domains from the four  $C_{\gamma 2}$  cDNAs and the homologous sequence of  $C_{\gamma 1}$ .

scripts, but fails to express Ti  $\alpha$ -chain messenger RNA. This finding suggested that a Ti  $\gamma/\beta$  heterodimer might be expressed on PEER. To assess this, the monoclonal antibody (mAb)  $\beta F1$ , which reacts specifically with all Ti  $\beta$ -chains<sup>9</sup>, was used in immunoprecipitation studies with PEER.

Although we failed to detect Ti  $\beta$  chains in lysates prepared from cell-surface iodinated PEER cells, a 41K Ti  $\beta$ -chain protein could be detected in lysates prepared from metabolically labelled cells (Fig. 1d and e). This indicates that Ti  $\beta$ -chains in PEER are not expressed on the cell surface. This excludes the possibility of Ti  $\beta/\gamma$  heterodimers on PEER.

All human Ti  $\gamma$ -chain cDNAs isolated to date have resulted from non-productive V-J rearrangements<sup>17,18</sup>. Therefore, isolation of an in-frame functional Ti  $\gamma$ -chain cDNA from PEER would further indicate that the 55K protein on PEER is a Ti  $\gamma$ -gene product. As the 55K protein on PEER is not expressed in the anticipated heterodimer configuration, examination of the primary Ti  $\gamma$ -chain sequence should be informative. A cDNA library of PEER was constructed in the  $\lambda$ gt10 phage vector. Of six Ti  $\gamma$ -chain cDNA clones isolated and sequenced, clones corresponding to three distinct transcripts were identified. Two clones, represented by P $\gamma 1$ , contain full-length sequences with in-frame rearrangements (Figs 2 and 3). Three other identical clones, represented by P $\gamma 12$ , are the result of an out-of-frame joining event (Fig. 2). A third clone, P $\gamma 11$ , corresponds to the product of a previously undescribed unrearranged V region pseudogene, a member of the V $\gamma 1$  subgroup (Fig. 2). This product may be analogous to upstream V region transcripts observed in the immunoglobulin (Ig) heavy-chain locus which can undergo secondary rearrangements resulting in V region replacements<sup>23,24</sup>. As in V $\mu$  regions, a consensus heptamer sequence (TACTGTG) is present at the 3' end of all human V $\gamma$  segments. As there is differential usage of V $\gamma$  genes during murine thymocyte development, it has been proposed that such upstream V $\gamma$  genes may be involved in secondary rearrangements<sup>25</sup>. The finding of a germline V $\gamma$  region transcript in PEER suggests that the Ti  $\gamma$  locus remains accessible for further rearrangements, and is consistent with this model.

Examination of the sequence of P $\gamma 1$  reveals several interesting features (Fig. 3). (1) This sequence is the result of in-frame joining of the V $\gamma 8$  gene segment (a member of the V $\gamma 1$  subgroup)

to J $\gamma 2$ <sup>16</sup>. Four additional nucleotides are found at the V-J junction; these are likely to be N nucleotides but may represent a previously undetected D region. (2) The constant region of this clone is nearly identical to the recently isolated  $C_{\gamma 2}$  cDNA<sup>18</sup> and, in contrast to products of the  $C_{\gamma 1}$  locus (see below), lacks cysteines external to the plasma membrane other than those in the immunoglobulin-like domain (Fig. 4). This is of particular interest as the 55K CD3-associated chain does not have a disulphide-linked partner chain, typically seen in Ti  $\alpha/\beta$  heterodimers. (3) The sequence contains five potential sites for N-linked glycosylation, compatible with the reported high degree of endoglycosidase F sensitivity of the 55K CD3-associated chain<sup>11</sup>. (4) Of considerable interest is a 48-base-pair (bp) triplication within the C region. Duplication of this 48-bp segment in the  $C_{\gamma 2}$  of HPB-MLT has been described<sup>18</sup>, and we confirmed the presence of this triplication in PEER by sequencing a second independent cDNA clone containing V $\gamma 8$  and found its C region sequence to be identical to P $\gamma 1$ .

A close examination of the 48-bp repeat of P $\gamma 1$  reveals that these 48-bp segments are not exact repeats but contain 2- or 5-bp differences, allowing discrimination between these repeated sequences (Fig. 4b). The C region found in the non-functionally rearranged Ti  $\gamma$  cDNA from PEER, P $\gamma 12$ , also contains a  $C_{\gamma 2}$ -like sequence with a duplication of 48-bp segments, but one segment is distinct from either of those described by Dialynas *et al.*<sup>18</sup>. We have isolated another Ti  $\gamma$  cDNA from the cell line FRO2.2 which also contains a 48-bp duplication identical to that of P $\gamma 12$ . All the duplicated 48-bp segments in these cDNA clones are represented in the triplicated segments seen in PEER (Fig. 4a). Thus, three distinct and very similar 48-bp sequences are observed and are variably used in the same or different cells as indicated in Fig. 4a.

The third segment in the triplication in P $\gamma 1$  is very similar to a single 48-bp segment contained within the constant region of a cDNA corresponding to the  $C_{\gamma 1}$  locus (Fig. 4); the only difference is a substitution of cysteine for tryptophan in the homologous segment of the  $C_{\gamma 1}$  gene. No other cysteines, excluding the two in the immunoglobulin-like region, are found external to the transmembrane domain in  $C_{\gamma 1}$ . In addition to the differences illustrated in Fig. 4, there are only five other amino-acid differences between products of the  $C_{\gamma 1}$  and  $C_{\gamma 2}$  loci (P. G. Pelicci *et al.*, in preparation).

The mechanism by which the distinct 48-bp repeats are variably expressed in  $C_{\gamma 2}$  is not readily apparent, but several explanations can be offered. Preliminary studies indicate that all three exist in the  $C_{\gamma 2}$  gene as separate exons which may have arisen recently by gene duplication (P. G. Pelicci *et al.*, in preparation). The variable expression of these 48-bp segments is likely to result from distinct alleles of the  $C_{\gamma 2}$  locus. Alternatively, although less likely, these segments may be variably used by alternative splicing or they may be present in other  $C_{\gamma}$  genes which remain to be detected. No comparable duplications have been described in the mouse, but these segments exhibit limited homology to a 69-bp segment in the recently-described murine  $C_{\gamma 4}$  gene, which is absent from the murine  $C_{\gamma 1-3}$  genes<sup>26</sup>. The explanation of the origin of these repeats awaits a more detailed analysis of the human Ti  $\gamma$  genomic organization.

The functional significance of these 48-bp repeats is unclear. A consequence of their presence in  $C_{\gamma 2}$  is the absence of a cysteine residue which is found within the highly homologous  $C_{\gamma 1}$  protein domain. This accounts for the failure of the Ti  $\gamma$  glycoprotein to form a disulphide-linked dimer on PEER and presumably other cells using  $C_{\gamma 2}$ . Whether the CD3-associated Ti  $\gamma$ -chain on PEER is a single chain or a non-disulphide-linked homodimer or heterodimer remains unclear. In view of the differences between  $C_{\gamma 1}$  and  $C_{\gamma 2}$  and of the variable expression of the 48-bp segments in  $C_{\gamma 2}$  sequences, it may be anticipated that different protein products of the Ti  $\gamma$ -chain locus will be observed. The non-disulphide-linked CD3 protein observed on PEER and the T-cell lines and the one thymocyte clone

reported<sup>9-11</sup> are likely to represent the expression of products of the  $C_{\gamma}2$  locus. Recent studies demonstrating CD3<sup>+</sup>, WT31<sup>-</sup> cells on which a disulphide linked 80-90K dimer is associated with CD3 may represent the expression of protein products of the  $C_{\gamma}1$  locus<sup>12,27</sup>. Further differences in  $C_{\gamma}2$  gene products are likely to be observed, as there are three potential *N*-linked glycosylation sites in the triplicated 48-bp segments; variable use of these 48-bp segments may result in substantial changes in molecular mass of the expressed Ti  $\gamma$ -chain protein. The ability of such different products of the  $C_{\gamma}$ -gene loci to function as receptors or as signal-transducing molecules may also differ.

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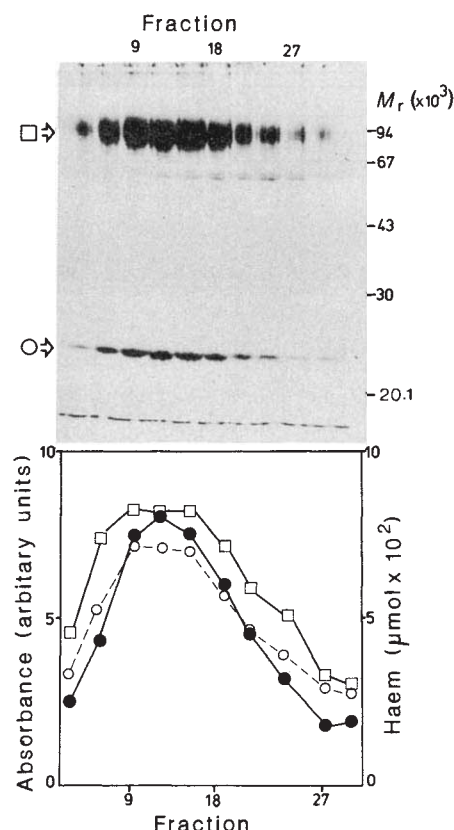
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## Absence of both cytochrome $b_{-245}$ subunits from neutrophils in X-linked chronic granulomatous disease

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Phagocytosis by neutrophils and other 'professional' phagocytic cells is accompanied by a microbicidal burst of non-mitochondrial respiration<sup>1</sup>. Cytochrome  $b_{-245}$  is the only clearly defined component of this oxidase system and its absence<sup>2</sup> provides the molecular basis of X-linked chronic granulomatous disease (CGD), in which a profound predisposition to infection results from complete failure of this respiratory burst<sup>3</sup>. Purification of the cytochrome has proved difficult, with uniform disagreement regarding the identity of its apoprotein, descriptions of its relative molecular mass ( $M_r$ ) on SDS-polyacrylamide gel electrophoresis (PAGE) ranging from 10,000 to 127,000 (10-127K)<sup>4-8</sup>. I report here that it has two subunits, a 23K protein and the previously described 76-92K glycoprotein<sup>9</sup>. These subunits are closely linked



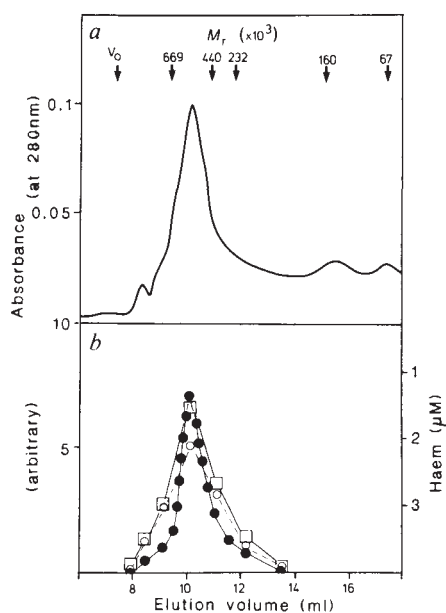
**Fig. 1** SDS-PAGE of fractions collected during the elution of highly purified cytochrome  $b_{-245}$  of neutrophils from a heparin-agarose affinity column. Two main bands of protein with apparent  $M_r$ s on gels of 76-92K ( $\square$ ) and 23K ( $\circ$ ) co-purify with the haemoprotein ( $\bullet$ ) of the cytochrome (below).

**Methods.** Neutrophils (400 ml packed cells) were fractionated on discontinuous sucrose gradients<sup>22</sup> and the membranes and specific granules, with a density of less than 1.21, were pelleted and extracted into buffer A<sup>4</sup> containing EDTA (1 mM pH 7.4), phenylmethylsulphonyl fluoride (1 mM), Trasylol (100,000 iu ml<sup>-1</sup>), tosyl-L-lysine chloromethyl ketone (TLCK, 27  $\mu$ M) as described<sup>4</sup>. After passing the extract sequentially through columns of CM and DEAE fast-flow Sepharoses (30 ml, Pharmacia Fine Chemicals) and *N*-amino-octyl-Sepharose (15 ml, Sigma) the cytochrome was bound to a column of heparin agarose (10 ml, Sigma) from which it was eluted with a gradient of NaCl (30 ml, 0.1-0.4 M) and collected in 1 ml fractions. An aliquot (100  $\mu$ l) from every third fraction was run on a 10% SDS-polyacrylamide gel with the Laemmli system of buffers<sup>23</sup> and stained with Coomassie blue. The peak specific activity (fraction 15) was 29.6 nmol haem per mg protein. The gel was scanned with a Chromoscan III scanner (Joyce Loebl). Comparison by regression analysis of the height of the peak of absorbance by the 76-92K band with that of the 23K band in the same track gave an  $r$  value of 0.99. The value was 0.94 when compared with the haem signal at 558 nm in the corresponding fraction.

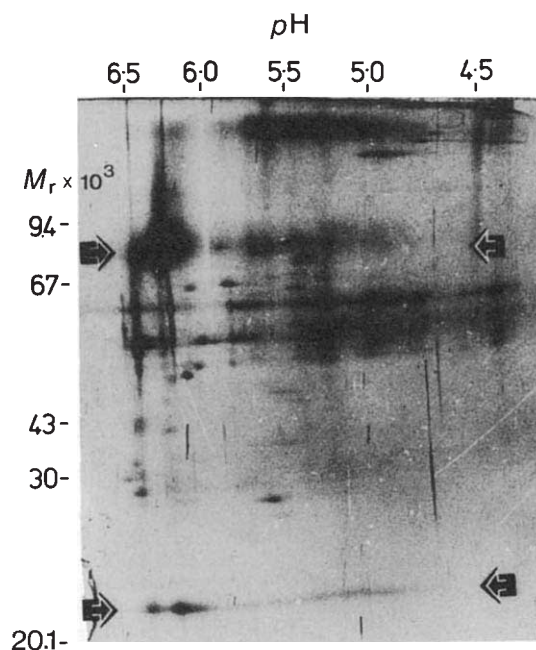
and remain associated with the haem of the cytochrome through affinity and gel filtration chromatography and sucrose gradient centrifugation, and exhibit a similar distribution in a pH gradient. Neither protein was detected in the cells of five patients with X-linked CGD whereas both were present in two with the form of this disease with autosomal recessive inheritance.

The molecular basis of the respiratory burst of neutrophils, monocytes, macrophages and eosinophils that is important for the killing<sup>10</sup> and digestion<sup>11</sup> of microbes by phagocytic cells has posed an interesting problem<sup>12</sup>. Attention was focused on cytochrome  $b_{-245}$  when it was identified as a component of this oxidase system and its absence defined as the molecular basis of the X-linked subtype of CGD<sup>2</sup>.





**Fig. 2** Gel filtration chromatography of cytochrome  $b_{-245}$  in which the 23K ( $\circ$ ) and 76-92K ( $\square$ ) proteins co-eluted with the haem of the cytochrome ( $\bullet$ ) with an apparent  $M_r$  of  $\sim 500$ –600K. **Methods.** Highly purified cytochrome  $b$  (6.8 nmol in 200  $\mu\text{l}$ ) was chromatographed in buffer A containing 1% Triton N101 on a FPLC Superose 12 column ( $1 \times 30$  cm, Pharmacia) at a flow rate of 0.1 ml  $\text{min}^{-1}$ . The peak of absorption at 280 nm, at a retention volume of 10.18 ml, coincided with the peaks of elution of the haem of the cytochrome, and of the two protein bands as measured by quantitative scanning of SDS-PAGE electrophoretograms. The size markers were blue dextran (void volume), thyroglobulin (669K), ferritin (440K), catalase (232K), immunoglobulin G (160K) and albumin (67K) which were chromatographed in buffer A in the absence of dithiothreitol.



**Fig. 3** Two-dimensional gel electrophoresis of cytochrome  $b_{-245}$ . The 23K and 76-92K proteins (arrows) showed a similar pattern of distribution in the pH gradient. **Methods.** Separation in the first dimension was by the method of O'Farrell<sup>24</sup>. An aliquot (200  $\mu\text{l}$ ) of fraction 15 from the preparation described in Fig. 1 was dialysed at 25  $^{\circ}\text{C}$  for 2 h against lysis buffer (9.5 M urea, 2% Nonidet P40, 5% 2-mercaptoethanol), applied to the acid end of the rod containing 2% Pharmalyte 3-10 (Pharmacia) and focused for 1,600 V h. The rod was then soaked in sample buffer<sup>23</sup> for 15 min, electrophoresed into the second dimension of 10% acrylamide, and silver stained<sup>13</sup>. Only the region of the gel in which the proteins were observed is shown.

Improvements in the purification of the cytochrome, by the use of additional protease inhibitors and more rapid separation techniques, enabled us to isolate a protein of  $M_r$  23K ( $23.49 \pm 8$ ,  $n = 7$ ) in addition to the customary broad protein band at 76-92K (s.d.s 8.4 and 5.3 respectively,  $n = 7$ , on 7.5–15% SDS-PAGE gels). Further studies were undertaken to examine the relationship of these two proteins to the cytochrome.

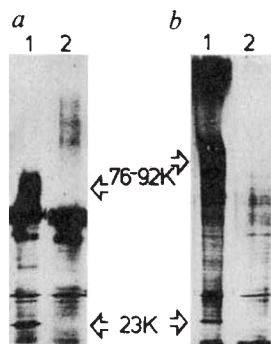
The association of the two proteins with each other and with the haem spectrum of cytochrome  $b_{-245}$  was determined when the latter was purified on heparin agarose (Fig. 1), and then subjected to gel filtration chromatography (Fig. 2). All three demonstrated an essentially identical elution profile from both columns. Assuming similar binding of Coomassie blue, the proteins appeared to be associated in roughly equimolar amounts. Essentially similar results were observed after the cytochrome was centrifuged into a linear gradient of sucrose (20–50%, containing 1% Triton N101, 130,000g for 20 h in a Sorvall TV-850 vertical rotor) in which it banded with a modal density of  $\sim 1.12$  (results not shown). Isoelectric focusing of the purified cytochrome in the presence of 8 M urea resulted in the same pattern of distribution of both proteins (Fig. 3). Although small amounts of both proteins were widely distributed in the first dimension, a large proportion of both focused with a  $pI$  of  $\sim 6.0$ –6.5.

The demonstration of the absence or abnormality of one or both of these proteins in X-linked CGD would provide strong additional evidence for their association with the cytochrome, the haem spectrum of which is missing in this condition<sup>2</sup>. I therefore modified our described method<sup>4</sup> to allow the purification of this cytochrome from the  $10^8$  cells readily obtainable from peripheral blood samples (Fig. 4 legend). This results

in a relatively high recovery of the cytochrome ( $44.5 \pm 19\%$ ,  $n = 4$ ) to a degree of purity ( $11.7 \pm 3.2$  nmol haem per mg protein,  $n = 4$ ) that compares well with the 5.3, 9–13, 16 and 20 nmol  $\text{mg}^{-1}$  reported for large-scale purifications (refs 5, 8, 4 and 6 respectively). After this purification the 23K and 76-92K proteins could be easily identified on SDS-PAGE electrophoretograms and as little as 10 pmol of the cytochrome (the amount present in  $10^6$  neutrophils) was clearly visible in a track after silver staining<sup>13</sup> the gel (Fig. 4).

The same procedure was then adopted with neutrophils from five male patients with X-linked CGD all of whose cells lacked spectral evidence of the cytochrome (patients nos 4, 14 and 19 in ref. 2, and two newly diagnosed boys with heterozygote carrier mothers). For comparison, two patients with an autosomal recessive pattern of inheritance of the disease, whose cells contained the cytochrome, a 20-year-old girl (patient no. 23, ref. 2) and a 41-year-old man, were also studied. In contrast to the presence of both these proteins in extracts from all ten normal individuals and the two patients with autosomal recessive CGD, both the 76-92K and the 23K proteins were undetectable in all five of the X-linked CGD patients (Fig. 4), clearly associating them with each other and with the cytochrome.

The purification of the complex of these two proteins almost certainly depends upon the binding of one, probably the 76-92K subunit<sup>4</sup>, to the heparin agarose. Thus both proteins might appear to be absent from cells lacking only that subunit carrying the requirements for purification. To examine this possibility, antibodies were raised to the 23K protein and used to probe Western blots of SDS-PAGE electrophoretograms of whole membranes. The antigen was detected in membranes from seven normal subjects and the female autosomal recessive patient, but



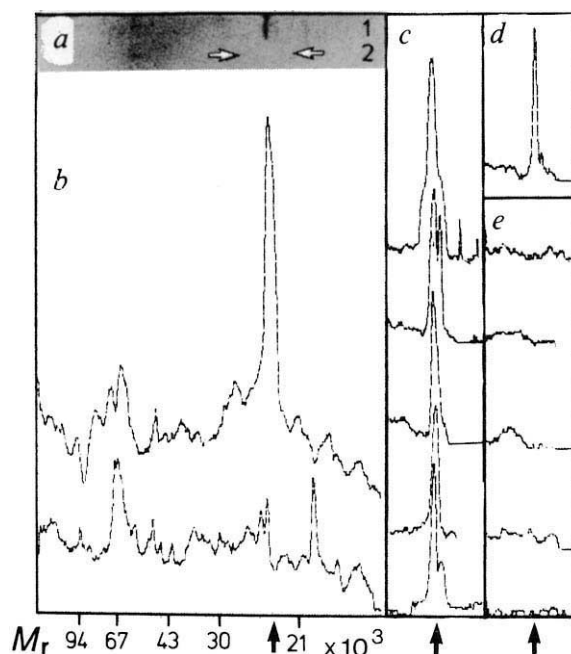
**Fig. 4** *a, b*, Two different comparisons of the pattern of proteins in extracts purified from two normal subjects (tracks 1 and 2) and two patients with X-linked CGD (tracks 1 and 2). The 76-92K and 23K protein bands (arrows) observed in extracts from normal cells are absent from those of the patients.

**Methods.** Neutrophils were purified from 100 ml venous blood<sup>25</sup> and pelleted at 600g at 20°C for 5 min. Diisopropyl fluorophosphate (5 µg) was added to the pellet and the cells were then resuspended in buffer A (see Fig. 1) without detergent and centrifuged at 80,000g at 4°C for 15 min. The cell pellet was extracted twice with sodium cholate (1% w/w) in buffer A and then washed in buffer A without detergent. The cytochrome was finally extracted twice in buffer A containing Triton N101 (1% w/w). Each time, the pellet was homogenized in the centrifuge tube in 8 ml of the appropriate buffer for 10 s at 24,000 r.p.m. with a Polytron homogenizer and centrifuged as described above. The Triton N101 extract was then passed (5 ml h<sup>-1</sup>) successively through: CM Sepharose; DEAE Sepharose; *N*-amino-octyl Sepharose; (1.5 ml bed volume of each resin) and then bound to heparin agarose (400 µl bed volume). The extract was followed with buffer A containing Triton N101 (10 ml at 5 ml h<sup>-1</sup>) after which the heparin agarose column was disconnected, washed with 5 ml of buffer A containing Triton N101 and NaCl (0.1 M) and the cytochrome was then eluted with the same buffer containing 0.7 M NaCl. The first 240 µl were discarded and the eluate was collected in 150 µl fractions, with the peak recovery of the cytochrome in the second and third fractions. The cytochrome was quantified from the reduced minus oxidized difference spectrum taking  $\Delta E_{559-540} = 21.6 \text{ cm}^{-1} \text{ mM}^{-1}$  for the  $\alpha$  band<sup>18</sup>. Protein was measured with the Pierce BCA reagent with bovine serum albumin as standard. Electrophoresis of all fractions was in 0.75-mm thick 5-15% slab gels which were silver stained<sup>13</sup>. The peak fraction of elution of the cytochrome from normal cells (fraction 3) is shown analysed in tracks 1 (21 and 16 pmol haem in tracks 1*a* and 1*b* respectively) and the corresponding fractions from the patients in tracks 2. The 23K and 76-92K proteins were not observed in any fractions from any of the five X-linked CGD patients.

not in those of all five X-linked patients studied (Fig. 5). Unfortunately these studies could not be extended to the 76-92K protein as we have been unable to raise suitable antisera, and antibodies against the 23K protein do not appear to immunoprecipitate the complex.

The active oxidase is thought to contain a flavoprotein<sup>14,15</sup> in addition to cytochrome *b*<sub>245</sub> but unfortunately the identification of their respective apoproteins has been hampered by the displacement of both the haem and flavin adenine dinucleotide (FAD) by mild denaturation. Hence, one of the subunits could be the apoprotein of the cytochrome and the other of the flavoprotein. It is interesting in this context that the FAD content of neutrophil membranes was determined in three of the five patients with X-linked CGD in this study, in all of whom it was found to be abnormally low<sup>14</sup>, although reduced levels of membrane-associated FAD has not been a consistent finding in this condition in the hands of other investigators<sup>16</sup>.

I propose that conventional terminology is adopted and the 23K and 76-92K proteins called the  $\alpha$  and  $\beta$  subunits respectively, assigning  $\beta$  to the larger subunit as it seems more probable that this is the haem-binding apoprotein: in our earlier



**Fig. 5** Immunoblot of neutrophil membranes using antiserum to the 23K protein (*a*) and reflectance scans of blots against normal subjects (*b-e*), confirming the absence of this protein from five patients with X-linked CGD (in *a*, track 2 (horizontal arrows)); in *b*, lower scan, and *e*, but not CGD with an autosomal recessive pattern of inheritance (*d*). A blot (*a*, upper track) and 6 scans from different normal subjects (*b*, upper scan, and *c*) are also shown. Arrows on the horizontal axis mark the position of the 23K protein.

**Methods.** Purified cytochrome *b*<sub>245</sub> (35 nmol haem, 2 mg protein) was subjected to SDS-PAGE on a 10% slab gel. The band of protein at 23K was electroeluted from the finely-chopped acrylamide in a dialysis sac in Tris/glycine/SDS<sup>23</sup> running buffer at 4°C at 320 V for 18 h. An aliquot of the eluted protein (50 µg) was inoculated with Freund's complete adjuvant by multiple subcutaneous injections into New Zealand White rabbits (2 kg body weight) followed by four further injections in Freund's incomplete adjuvant at two-weekly intervals. Membrane proteins (*a, b*, 40 µg; *c-e*, 20 µg per track) were run on a 12.5% gel and blotted onto nitrocellulose for 3 h at 550 mA in running buffer containing 20% methanol. The blot was washed in Tris/HCl (50 mM, pH 7.4) buffered NaCl (200 mM) containing Tween 20 (0.05%) (TBS/Tween 20) for 10 min, blocked with bovine serum albumin (3%) and normal goat serum (10%) in TBS/Tween 20 for 10 min and incubated at room temperature in the antiserum (1 in 25 dilution in blocking buffer) for 2 h, washed in TBS/Tween for 10 min, washed for 10 min in block buffer, incubated with goat anti-rabbit IgG (H & L) horseradish peroxidase conjugate (Biorad, Western Blotting Grade, 1 in 2,000 in block buffer) for 1 h at 25°C, washed for 5 min in TBS/Tween and developed with 4-chloro-1-naphthol (2.8 mM) and H<sub>2</sub>O<sub>2</sub> (4.4 mM) in TBS at 25°C. Minor coloured bands were sometimes observed at about 60K and 18K in the absence of the first antiserum.

purifications of the cytochrome it was seen as a single band<sup>4</sup>. The gel filtration studies indicated an *M<sub>r</sub>* for the cytochrome of ~500-600K. Although the anomalous behaviour of membrane proteins in detergent on gel filtration columns makes accurate predictions of molecular size impossible<sup>17</sup>, it would not be surprising if these subunit assemblies were arranged in dimers or tetramers. Although such quaternary structure would be very unusual for a mammalian cytochrome *b*, we know this cytochrome to be atypical, resembling bacterial cytochromes *o*<sup>18</sup> which have between 1 and 4 subunits<sup>19</sup>.

Orkin's group recently cloned a gene on the X chromosome<sup>20</sup> that they found to be abnormal in X-linked CGD. It appears to encode a protein that appears different from both subunits



of cytochrome  $b_{-245}$ : it has a dissimilar amino-acid composition from the  $\beta$  subunit<sup>9</sup> and its predicted  $M_r$  of 54K is more than twice that of the  $\alpha$  subunit. It could be another redox component of the oxidase system, although it does not have an obvious haem<sup>20</sup> or nucleotide<sup>21</sup> binding site, or an associated structural protein. If, as they claim, the gene they have cloned is the site of the primary lesion in X-linked CGD, the identification of the gene product in question will be required in order to understand

its relationship to the phenotypic absence of the protein subunits of cytochrome  $b_{-245}$  described here.

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## Linkage of an X-chromosome cleft palate gene

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Many congenital malformations, such as cleft palate and neural tube defects, have a multifactorial origin involving both environmental and genetic factors. Conditions such as these may be exclusively monogenic, polygenic or environmental, but in most cases both genetic and environmental factors are involved<sup>1</sup>. This study describes the sub-chromosomal localization of a single gene defect causing cleft palate and ankyloglossia (tongue-tied) in a large Icelandic family. This defect is a model for the analysis of other neural-crest malformations that show a more complex multifactorial inheritance pattern.

Human secondary cleft palate (CP) occurs during the seventh to ninth weeks of embryological development when the lateral palatine shelves fail to fuse<sup>2</sup>. This malformation shows little variation between populations; the incidence is ~1 in 1,500<sup>3</sup>. CP is more prevalent in females than in males<sup>4</sup>, which may be because the palatine processes fuse one week later in females<sup>5</sup>, allowing more time for teratogenic agents to play a part in the potential for malformation in females.

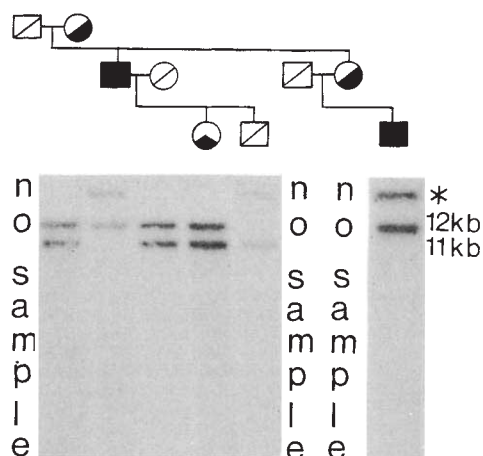
The analysis of single gene mutations using restriction fragment length polymorphisms (RFLPs) for linkage studies has been successful in determining the chromosomal location of several common inherited disorders. Linkage to within 5 centimorgans has been detected, both in cases where the affected chromosome is known, as for the sex-linked disorders Duchenne muscular dystrophy<sup>6</sup> and chronic granulomatous disease<sup>7</sup>, and when neither the autosome nor the biochemical defect is identified, as for Huntington's disease<sup>8</sup> and cystic fibrosis<sup>9-11</sup>. However, the analysis of polygenic disorders by linkage studies with RFLP markers is at present more complex both theoretically and practically.

One way to learn more about the aetiology of disorders with complex combinations of genetic and environmental factors is to use as a model a family in which the phenotype is due to a single gene defect, but displays the same features as more common multifactorial sporadic cases. Such a model for mid-line congenital defects has been found in a large Icelandic family

**Table 1** X-chromosome probes that show linkage to the CP+A locus: their localization, polymorphism and lod scores at various recombination fractions ( $\theta$ ).

| Probe  | X-chromosome location | Polymorphism-enzyme | lod at $\theta$ value |       |      |      |      |      |      |
|--------|-----------------------|---------------------|-----------------------|-------|------|------|------|------|------|
|        |                       |                     | 0                     | 0.01  | 0.05 | 0.10 | 0.20 | 0.30 | 0.40 |
| L1.28  | p11.3                 | TaqI                | 0.38                  | 0.37  | 0.36 | 0.33 | 0.27 | 0.19 | 0.10 |
| p58.1  | p11-cen               | MspI                | 0.65                  | 0.65  | 0.64 | 0.62 | 0.52 | 0.38 | 0.21 |
| cpX203 | p1.1                  | BglII               | —∞                    | -0.33 | 0.22 | 0.40 | 0.46 | 0.37 | 0.21 |
| pDP34  | q13-q21               | TaqI                | 3.07                  | 3.02  | 2.81 | 2.53 | 1.92 | 1.28 | 0.62 |
| X65H7  | q13-q22               | HindIII             | —∞                    | -0.03 | 0.92 | 1.06 | 0.97 | 0.71 | 0.38 |
| 7b     | q13-q22               | PstI                | —∞                    | -0.53 | 0.18 | 0.37 | 0.44 | 0.36 | 0.21 |

All likelihoods and lod scores were calculated with the LINKAGE computer program package<sup>20</sup>. Individuals with either cleft palate and/or ankyloglossia were considered to be uniformly affected (that is both phenotypes combined to one 'affection status') and the maximal likelihood estimate for the penetrance ( $t$ ) of this single gene for 'carrier females' was calculated to be 82%. The lod scores ( $Z$ ) were calculated according to Ott<sup>21</sup>:  $Z(\theta) = \log \text{likelihood}(\theta, t) - \log \text{likelihood}(1/2, t)$ , where  $t = 0.82$ . The frequency for the CP+A mutant allele was taken as 0.0001. Likelihoods calculated at a range of penetrances, RFLP and CP+A allele frequencies made no significant difference to the quoted lod scores (data available on request). Respectively 3, 6, 4, 8, 6 and 5 meioses contributed most of the linkage data for probes L1.28, p58.1, cpX203, pDP34, X65H7 and 7b (for probe localization see ref. 22).



**Fig. 1** *TaqI*-digested DNA samples from the pedigree are shown hybridized to the anonymous linked pDP34 marker. Section of the Icelandic family showing the X-linked inheritance of CP and A (□ or ○, unaffected ♂ and ♀; ■, affected ♂ CP+A; ●, affected ♀, A alone; ◐, obligatory carrier; \*, Y-specific band).

**Method.** Genomic DNA was prepared from 10 ml of whole blood<sup>23</sup>. DNA (4 µg) was digested with the relevant restriction enzyme revealing the polymorphism for each X-chromosome probe. The digested DNA was fractionated by electrophoresis on 1% agarose gels and transferred to Hybond-N membranes (Amersham)<sup>24</sup>. The probes used were labelled with a specific activity of  $1 \times 10^9$  d.p.m. µg<sup>-1</sup> by synthesis using random oligonucleotide primers. The filters were washed down to a final salt concentration of  $0.1 \times \text{SSC}$  (SSC is 0.15 M NaCl, 0.015 M sodium citrate) in the presence of 0.1% SDS at 65 °C. Autoradiography was for 24 h at -70 °C with an intensifying screen.

(over 200 members) showing mendelian inheritance of X-linked secondary cleft palate and ankyloglossia ('tongue-tied') (CP+A). Both the large size of this pedigree and the availability of many defined X-chromosome probes has made it possible to localize this defect sub-chromosomally.

A section of the pedigree of 293 individuals in the family studied is shown in Fig. 1; three of us (A.B., A.A. and O.J.) have personally investigated 182 individuals in four living generations, and information on other family members was gained either from written or family records<sup>12</sup>. (Full pedigree data are available from the authors on request.) Blood was collected from 82 individuals for RFLP genotyping, including 9 males with secondary cleft palate and ankyloglossia (CP+A) and 10 females with ankyloglossia alone (A). One female had CP alone and one male had a patent but high-vaulted palate (HVP) characteristic of those affected. For purposes of linkage analysis

each family member was designated affected if they had any of the features diagnostic for CP, A or HVP.

Of the 14 matings where a CP+A father has the opportunity to pass a mutant gene to his son, the son is never affected. This suggests that this gene is sex-linked. Occasionally, an unaffected female has affected sons and daughters, and is postulated to be a carrier; the maximal likelihood estimate for the penetrance of the mutation is calculated to be 82% (Table 1).

The analysis of inheritance of RFLPs was carried out using standard techniques (Fig. 1). Linkage at a lod score of 3.07 has been demonstrated between the CP+A locus and the locus *DXYS1* of the anonymous DNA probe pDP34, which maps to Xq13-Xq21<sup>13</sup> (Table 1). Further linkage analysis of the loci defined by an additional 14 informative probes has allowed exclusion of most of the rest of the X chromosome.

Localization of the mutation causing cleft palate in this family is a first step in understanding the genetic component of congenital neural tube and crest defects. This region of the X chromosome contains an X-Y homologous region<sup>14</sup>; the availability of many X-Y homologous probes should permit the fine mapping of the defect. As the limits of genetic mapping in this family are approached the techniques of cosmid walking and jumping and pulsed-field gel electrophoresis<sup>15,16</sup> will allow the gap between the genetic and physical map to be bridged.

The isolation of the gene and studies of its spatial and temporal expression during development of the secondary palate should lead us to a greater understanding of mechanisms which may lead to the failure of closure of the neural crest. This mechanism can be compared with those which may cause sporadic cases in humans, or in animal models. There are also families known in which an X-linked form of spina bifida and anencephaly occur<sup>17</sup>; any homology in linkage between these families and the Icelandic family would be of interest.

Many disorders have both genetic and environmental elements. Polygenic and/or multifactorial disorders are complex to analyse, as has been elegantly shown for the role of LDL receptor defects in atherosclerosis<sup>18</sup>. Eventual cloning of the 'cleft palate' gene could be used as a starting point for the analysis of the genetic element of this developmental abnormality. For example, this gene may be a member of a multigene family<sup>19</sup> involved in the embryonic development of other tissues. Analysis of CP+A as a sex-linked single gene could provide a model for identifying other genes regulating processes in embryonic development whose expression is usually hidden in phenotypic, polygenic complexity.

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# The molecular clock runs more slowly in man than in apes and monkeys

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The molecular clock hypothesis<sup>1</sup> postulates that the rate of molecular evolution is approximately constant over time. Although this hypothesis has been highly controversial in the past, it is now widely accepted<sup>2-5</sup>. The assumption of rate constancy has often been taken as a basis for reconstructing the phylogenetic relationships among organisms or genes and for dating evolutionary events<sup>2-5</sup>. Further, it has been taken as strong support for the neutral mutation hypothesis<sup>5</sup>, which postulates that the majority of molecular changes in evolution are due to neutral or nearly neutral mutations<sup>6</sup>. For these reasons, the validity of the rate constancy assumption is a vital issue in molecular evolution. Recent studies<sup>7-12</sup> using DNA sequence data have raised serious doubts about the hypothesis. These studies provided support for the suggestion made from immunological distance and protein sequence data<sup>13,14</sup> that a rate slowdown has occurred in hominoid evolution, and showed, in agreement with DNA hybridization studies<sup>15,16</sup>, that rates of nucleotide substitution are significantly higher in rodents than in man. Here, rates of nucleotide substitution in rodents are estimated to be 4–10 times higher than those in higher primates and 2–4 times higher than those in artiodactyls. Further, this study provides strong evidence for the hominoid slowdown hypothesis<sup>13,14</sup> and suggests a further rate-slowdown in hominoid evolution. Our results suggest that the variation in rate among mammals is primarily due to differences in generation time<sup>8,16</sup> rather than changes in DNA repair mechanisms<sup>9</sup>. We also propose a method for estimating the divergence times between species when the rate constancy assumption is violated.

Wu and Li<sup>8</sup> estimated that since the mammalian radiation the rate of synonymous substitution has been, on average, about two times higher in the rodent lineage than in the human and artiodactyl lineages. Because at the early stage of mammalian divergence the substitution rates in the three lineages should have been very similar, the rate differences noted by Wu and Li must have occurred in more recent times. To see if this is true we have compared the rates of synonymous substitution in primates, rodents and artiodactyls (Table 1). We used the method of Li *et al.*<sup>17</sup> to estimate the number of substitutions per synonymous site ( $K_S$ ) and per nonsynonymous site ( $K_A$ ) between two homologous genes. In this method, a nucleotide site is counted as one synonymous site if all possible changes at that site are synonymous, and  $\frac{1}{3}$  or  $\frac{2}{3}$  if one or two of the three possible changes are synonymous.

In the comparison between man and chimpanzee (Table 1), the synonymous rate varies considerably among the genes studied. This is probably largely due to chance effects, because the number of synonymous sites in each gene is small and the degree of sequence divergence is low. Further, the higher rates in the  $\alpha 1$ -,  $\gamma$ - and  $\Lambda$ -globin genes might be partly due to gene conversions between the two  $\alpha$ -globin genes<sup>18,19</sup> and between the two  $\gamma$ -globin genes<sup>20,21</sup>. Despite this possibility, the average rate for the five genes is only about  $1.1 \times 10^{-9}$  substitutions per synonymous site per year. In the comparison between man and the Old World (OW) monkeys, the synonymous rates are quite uniform over genes except for the insulin gene, and the average rate is about  $2.3 \times 10^{-9}$ . This is twice the average rate for the human-chimpanzee divergence, suggesting that OW monkeys have evolved two times faster than higher primates. The average rate ( $4 \times 10^{-9}$ ) between cow and goat seems to be substantially higher than that in higher primates, although the number of genes used is small. The rate estimated from the ruminant  $\epsilon$

**Table 1** Rates of synonymous substitution per site per year in primates, rodents and artiodactyls

| Gene                                                     | No. of sites | Divergence (%) | Rate ( $\times 10^{-9}$ ) |
|----------------------------------------------------------|--------------|----------------|---------------------------|
| Man vs chimpanzee: divergence time used = 7 (5–10) Myr   |              |                |                           |
| $\alpha 1$ -Globin                                       | 108          | 2.8            | 2.0 (1.4–2.8)             |
| $\alpha 2$ -Globin                                       | 108          | 0.0            | 0.0 (0.0–0.0)             |
| $\beta$ -Globin                                          | 89           | 0.0            | 0.0 (0.0–0.0)             |
| $\gamma$ -Globin                                         | 101          | 3.0            | 2.1 (1.5–3.0)             |
| $\Lambda$ -Globin                                        | 101          | 2.0            | 1.4 (1.0–2.0)             |
| Total                                                    | 507          | 1.6            | 1.1 (0.8–1.6)             |
| Man vs OW monkeys: divergence time used = 25 (20–30) Myr |              |                |                           |
| $\alpha 1$ -Antitrypsin                                  | 270          | 11.1           | 2.2 (1.9–2.8)             |
| Erythropoietin                                           | 145          | 11.2           | 2.2 (1.9–2.8)             |
| $\delta$ -Globin                                         | 104          | 10.4           | 2.0 (1.7–2.6)             |
| Insulin                                                  | 84           | 18.6           | 3.7 (3.1–4.7)             |
| $\beta$ -Globin (partial)                                | 72           | 8.9            | 1.8 (1.5–2.2)             |
| Metallothionein II                                       | 35           | 9.1            | 1.8 (1.5–2.3)             |
| Total                                                    | 710          | 11.6           | 2.3 (1.9–2.9)             |
| Cow vs goat: divergence time used = 17 (12–25) Myr       |              |                |                           |
| $\beta$ -Globin                                          | 99           | 13.6           | 4.0 (2.7–5.7)             |
| $\gamma$ -Globin                                         | 105          | 13.5           | 4.0 (2.7–5.7)             |
| Mouse vs rat: divergence time used = 15 (10–30) Myr      |              |                |                           |
| Aldolase A                                               | 184          | 15.4           | 5.1 (2.6–7.7)             |
| Creatine kinase M                                        | 251          | 17.2           | 5.7 (2.9–8.6)             |
| Apolipoprotein E                                         | 201          | 17.4           | 5.8 (2.9–8.7)             |
| THY-1                                                    | 116          | 19.3           | 6.4 (3.2–9.6)             |
| Metallothionein I                                        | 37           | 11.5           | 3.8 (1.9–5.7)             |
| LDH-A                                                    | 219          | 30.9           | 10.3 (5.2–15.5)           |
| GPHA                                                     | 58           | 30.8           | 10.3 (5.1–15.4)           |
| ANF                                                      | 107          | 20.4           | 6.8 (3.4–10.2)            |
| Growth hormone                                           | 124          | 14.1           | 4.7 (2.4–7.1)             |
| Prolactin                                                | 127          | 21.9           | 7.3 (3.7–11.0)            |
| $\alpha$ -Actin                                          | 249          | 17.9           | 6.0 (3.0–8.9)             |
| POMC                                                     | 154          | 21.4           | 7.1 (3.6–10.7)            |
| Thyrotropin, $\beta$                                     | 90           | 25.7           | 8.6 (4.3–12.8)            |
| $\alpha$ -Fetoprotein                                    | 321          | 37.2           | 12.4 (6.3–18.9)           |
| Albumin                                                  | 274          | 36.7           | 12.2 (6.1–18.3)           |
| Total                                                    | 2,511        | 23.7           | 7.9 (4.0–11.9)            |

In the case of cow versus goat, the rate for  $\beta$ -globin is the average for the comparisons between cow  $\beta$ -globin gene and goat  $\beta^A$ - and  $\beta^C$ -globin genes. As divergence times are usually now well established, we have considered a range of estimates and an intermediate date for every pair of species compared. References: for the human-chimpanzee split<sup>24,32</sup>; the human/Old World monkey split<sup>25,26</sup>; the cow-goat split<sup>29–31</sup>; the mouse-rat split<sup>4,38</sup>. Data sources:  $\alpha$ -globin<sup>18,19</sup>;  $\beta$ -globin<sup>23,39</sup>; erythropoietin<sup>40</sup>; THY-1 (ref. 41);  $\alpha$ -fetoprotein and albumin<sup>33,42</sup>; LDH-A (references in ref. 43); aldolase A<sup>44,45</sup>; creatine kinase M<sup>46,47</sup>; thyrotropin $\beta$ <sup>23,48</sup>. For other sequences, see ref. 23.

genes is even higher ( $4.8 \times 10^{-9}$ ), but these genes might have been involved in gene conversion events<sup>22</sup>. In the comparison between mouse and rat, except for the  $\alpha$ -fetoprotein and albumin genes and some short sequences, the synonymous rates lie between  $6 \times 10^{-9}$  and  $10 \times 10^{-9}$ . It is seen that even the lower estimates of the synonymous rates in rodents are generally higher than the upper estimates in higher primates and monkeys. The intermediate estimate for the synonymous rate in rodents is 7 times higher than that in higher primates. This is similar to Britten's<sup>9</sup> estimate of a fivefold difference, derived mainly from DNA hybridization data. Considering the results in Table 1 and in Wu and Li<sup>8</sup> we suggest that the average synonymous rate in rodents is 4–10 times higher than that in higher primates, 3–6 times higher than that in monkeys, and 2–4 times higher than that in artiodactyls.

The results in Table 1 suggest a higher rate in artiodactyls than in primates. This is further supported by pseudogene data. For the  $\alpha$ - and  $\eta$ -globin pseudogenes<sup>11,23</sup> (2,773 base pairs (bp)) the sequence divergence between human and chimpanzee is 1.7%, which corresponds to a rate of  $1.2 \times 10^{-9}$  if we assume

**Table 2** Differences in the number of nucleotide substitutions per 100 sites between a primate (species 1) lineage and the human (species 2) lineage

| Sequence           | Sites* compared | NW monkeys |                       | OW monkeys |                        | Orangutan |                   | Gorilla  |                        | Chimpanzee |                   |
|--------------------|-----------------|------------|-----------------------|------------|------------------------|-----------|-------------------|----------|------------------------|------------|-------------------|
|                    |                 | $K_{12}$   | $K_{13} - K_{23}$     | $K_{12}$   | $K_{13} - K_{23}$      | $K_{12}$  | $K_{13} - K_{23}$ | $K_{12}$ | $K_{13} - K_{23}$      | $K_{12}$   | $K_{13} - K_{23}$ |
| $\eta$ -Pseudogene | 2,000           | 11.2       | 2.4 $\dagger \pm 1.1$ | 7.3        | 2.1 $\ddagger \pm 0.7$ | 3.1       | 0.4 $\pm 0.4$     | 1.4      | 0.5 $\pm 0.27$         | 1.3        | 0.5 $\pm 0.27$    |
| Synonymous         |                 |            |                       |            |                        |           |                   |          |                        |            |                   |
| $\gamma$ -Globin   | 100             | 13.6       | -3.7 $\pm 6.8$        |            |                        |           |                   | 0.0      | 0.0                    | 2.5        | 1.8 $\pm 1.60$    |
| $\beta$ -Globin    | 70              |            |                       | 7.6        | 1.2 $\pm 6.0$          |           |                   |          |                        |            |                   |
| EPO                | 145             |            |                       | 11.2       | 6.8 $\pm 5.9$          |           |                   |          |                        |            |                   |
| $\alpha$ 1-AT      | 140             |            |                       | 10.9       | 6.7 $\pm 8.9$          |           |                   |          |                        |            |                   |
| Insulin            | 84              |            |                       | 18.6       | -7.5 $\pm 6.9$         |           |                   |          |                        |            |                   |
| MT-II              | 35              |            |                       | 9.1        | 3.6 $\pm 8.2$          |           |                   |          |                        |            |                   |
| $\alpha$ -Globin   | 105             |            |                       |            |                        | 6.9       | 3.2 $\pm 4.5$     |          |                        | 1.4        | 0.1 $\pm 2.96$    |
| Introns            |                 |            |                       |            |                        |           |                   |          |                        |            |                   |
| $\gamma$ -Globin   | 900             | 11.6       | 3.1 $\pm 1.6$         |            |                        |           |                   | 3.0      | 0.3 $\pm 0.66$         | 2.7        | 0.0 $\pm 0.60$    |
| UT regions         |                 |            |                       |            |                        |           |                   |          |                        |            |                   |
| $\gamma$ -Globin   | 255             | 15.3       | 3.9 $\pm 3.5$         |            |                        |           |                   | 1.2      | 0.3 $\pm 0.75$         | 0.8        | 0.0 $\pm 0.37$    |
| $\beta$ -Globin    | 180             |            |                       | 3.9        | 1.8 $\pm 1.5$          |           |                   |          |                        | 0.0        | 0.0               |
| $\alpha$ -Globin   | 140             |            |                       |            |                        | 4.3       | 1.1 $\pm 3.5$     |          |                        | 3.9        | 1.2 $\pm 3.41$    |
| mtDNA              |                 |            |                       |            |                        |           |                   |          |                        |            |                   |
| Fragment           | 895             |            |                       |            |                        | 18.0      | 0.9 $\pm 1.7$     | 11.1     | 0.9 $\pm 1.31$         | 9.4        | 1.4 $\pm 1.21$    |
| 12S RNA            | 955             |            |                       |            |                        |           |                   | 3.6      | 0.2 $\pm 0.80$         | 3.8        | 0.4 $\pm 0.80$    |
| Total (nuclear)    |                 | 11.6       | 2.6 $\dagger \pm 0.9$ | 6.9        | 2.1 $\ddagger \pm 0.6$ | 3.2       | 0.4 $\pm 0.4$     | 1.6      | 0.5 $\dagger \pm 0.24$ | 1.3        | 0.3 $\pm 0.20$    |
| No. of sites       |                 | 2,920      |                       | 2,730      |                        | 2,210     |                   | 3,170    |                        | 3,520      |                   |

The relative rate test<sup>32</sup> was used. In the case of pseudogenes and noncoding regions, the method of Jukes and Cantor<sup>49</sup> and Kimura and Ohta<sup>50</sup> was used to estimate the mean ( $K_{ij}$ ) and variance ( $V_{ij}$ ) of the number of substitutions per site between sequences  $i$  and  $j$ ;  $i, j = 1, 2$  or  $3$ , where  $3$  refers to the reference species. Let  $K_{03} = (K_{13} + K_{23} - K_{12})/2$ ,  $P = 0.75[1 - \exp(K_{03}/0.75)]$  and  $V_{03} = P(1 - P)/[L(1 - P/0.75)^2]$ , where  $L$  is the number of sites compared. Then the variance of  $K_{13} - K_{23}$  is given by  $V_{13} + V_{23} - 2V_{03}$  (See ref. 8 for the derivation). In the case of synonymous substitutions,  $K_{ij}$  and  $V_{ij}$  were estimated by the method of Miyata *et al.*<sup>51</sup>, and the variance of  $K_{13} - K_{23}$  was computed according to the above procedure. In the case of New World (NW) monkeys versus human, the reference species was goat ( $\eta$ -pseudogene) or rabbit ( $\gamma$ -globin). In the case of Old World (OW) monkeys vs human, the reference species was owl monkey ( $\eta$ -pseudogene), cow and rabbit ( $\beta$ -globin), mouse (EPO and  $\alpha$ 1-AT), dog (insulin), or chinese hamster (MT-II). In the case of orangutan versus human, the reference species were owl monkey and rhesus monkey ( $\eta$ -pseudogene), rabbit ( $\alpha$ -globin), or gibbon (mitochondrial (mt) DNA fragment). In the case of gorilla (or chimpanzee) versus human, the reference species were owl monkey, rhesus monkey and orangutan ( $\eta$ -pseudogene), spider monkey ( $\gamma$ -globin), orangutan ( $\alpha$ -globin, mtDNA fragment and 12S RNA), or rhesus monkey ( $\beta$ -globin untranslated (UT) regions). When more than one species was used as a reference in a test, the smallest value for the variance of  $K_{13} - K_{23}$  was chosen; this still tends to overestimate the variance. 'Total' mean and variance were computed by using the reciprocal of the variance of  $K_{13} - K_{23}$  as weight. Data sources:  $\gamma$ -globin<sup>7,10,20,21,52</sup>;  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT)<sup>23,53</sup>;  $\alpha$ -globin<sup>23,54,55</sup>; mtDNA fragment<sup>56</sup>, and mt 12S RNA<sup>57</sup>. For the other sequences, see Table 1 and ref. 23.

\* Approximate number of sites compared.  $\dagger$  Significant at the 5% level.  $\ddagger$  Significant at the 1% level.

**Table 3** Mean (below diagonal) and standard error (above diagonal) of the number of nucleotide substitutions between species per 100 sites in the  $\eta$ -globin pseudogene

| Species    | Human | Chimpanzee | Gorilla | Orangutan | Rhesus | Owl monkey | Goat |
|------------|-------|------------|---------|-----------|--------|------------|------|
| Human      | —     | 0.26       | 0.27    | 0.40      | 0.63   | 0.80       | 1.75 |
| Chimpanzee | 1.33  | —          | 0.32    | 0.43      | 0.66   | 0.82       | 1.75 |
| Gorilla    | 1.39  | 1.96       | —       | 0.45      | 0.66   | 0.81       | 1.76 |
| Orangutan  | 3.11  | 3.59       | 3.81    | —         | 0.65   | 0.81       | 1.77 |
| Rhesus     | 7.31  | 7.87       | 7.82    | 7.76      | —      | 0.88       | 1.85 |
| Owl monkey | 11.16 | 11.64      | 11.58   | 11.46     | 13.26  | —          | 1.83 |
| Goat       | 35.11 | 35.11      | 35.59   | 35.68     | 38.20  | 37.52      | —    |

Data from ref. 11 and references therein.

that human and chimpanzee diverged 7 Myr ago<sup>24</sup>. The divergence between the rhesus monkey and owl monkey  $\eta$ -globin pseudogenes<sup>11</sup> (1,967 bp) is 13.3%, corresponding to a rate of  $1.7 \times 10^{-9}$  if we assume 40 Myr for the divergence between the rhesus and owl monkeys<sup>25-27</sup>. For the comparisons between cow  $\psi^3\beta$  and goat  $\psi^3\beta^X$  and  $\psi^3\beta^Z$  globin pseudogenes<sup>23,28</sup> (1,212 bp) the divergence is 9.1%. If we assume 17 Myr for the cow-goat split,<sup>29-31</sup> this corresponds to a rate of  $2.7 \times 10^{-9}$ , about 2.3 times higher than the rate in higher primates and 1.5 times higher than the rate in monkeys.

We now turn to the controversial issue of whether there has been a rate slowdown in hominoid evolution<sup>4,13,14,24,32</sup>. We use the relative rate test (see Table 2, footnotes). In Table 2, lineage 2 is always the human lineage whereas lineage 1 is either a monkey or an ape lineage. Therefore, a positive sign means that the human lineage has evolved more slowly and a negative sign means the opposite. It is striking that a negative sign appears

in only two of the 30 comparisons (Table 2). Evidently, there is a general trend towards a slower rate in the human lineage. In the comparison between the New World (NW) monkey and the human lineages the difference in rate is significant (at the 5% level) in the case of the  $\eta$ -globin pseudogene and is close to significance in the case of  $\gamma$ -globin introns. The difference becomes highly significant when all sequences are considered together. The number of nucleotide substitutions per 100 sites is  $7.1 = (11.6 + 2.6)/2$  in the NW monkey lineage and  $4.5 = (11.6 - 2.6)/2$  in the human lineage. Thus, the rate is about 1.6 times faster in the former than in the latter lineage. In the comparison between the OW monkey and human lineages the difference in rate is highly significant even when only the  $\eta$  pseudogene is used. When all sequences are considered together, the rate is  $1.9 (= 4.5/2.4)$  times faster in the OW monkey lineage than in the human lineage, similar to the factor of two derived above from synonymous substitutions (Table 1).



The rate in the human lineage seems also to be lower than those in the ape lineages (Table 2). In all the comparisons between an ape lineage and the human lineage, there is no case in which the human lineage has evolved faster. This is true for both nuclear and mitochondrial sequences. When all the nuclear sequences are considered together, the rates in the orangutan, gorilla and chimpanzee lineages are, respectively, 1.3, 1.9 and 1.6 times faster than the rate in the human lineage.

For  $\eta$ -globin, comparisons can also be made between apes and monkeys (Table 3). If we use owl monkey as a reference, it becomes clear that the rate is slower in the apes than in rhesus monkey because the distances from the former species to owl monkey are shorter than that from rhesus monkey to owl monkey. If goat is used as a reference, it also becomes clear that the rate is slower in the apes than in owl monkey. These results, together with those in Table 2, suggest that the substitution rate has become progressively slower in the human lineage since the separation of man from the OW monkeys.

As the rate constancy assumption is often violated, we need to consider the possibility of unequal rates among lineages when estimating the divergence time between species or genes. This can be done as follows, using the  $\eta$ -globin pseudogenes from primates as an example. Suppose that the branching order for the five primate species in Table 3 is as shown in Fig. 1, which is also the branching order inferred by either the maximum parsimony method<sup>11</sup> or Li's method<sup>33</sup>. The branch lengths are computed using a procedure that takes into account unequal substitution rates (Fig. 1 legend).

Suppose that we know the branching date for orangutan, say 12 Myr BP (ref. 34), and want to estimate the branching dates for gorilla and chimpanzee. The distances from node *b* to orangutan, gorilla and chimpanzee are 1.86, 1.95 and 1.91, respectively. Therefore, the rate constancy assumption holds approximately and the two branching dates are estimated to be  $12 \times 1.00/1.95 = 6.2$  Myr and  $6.2 \times 0.92/0.96 = 5.9$  Myr. Note that we avoid using the human lineage because the rate in that lineage has obviously been greatly reduced. As another example, suppose that we know the branching date for rhesus monkey, say 25 Myr (refs 25 and 26), and want to estimate the branchpoint for orangutan. Assuming that the substitution rate has been constant in the orangutan lineage since its separation from rhesus monkey, we estimate the orangutan branch point to be  $25 \times 1.86/(1.86 + 1.12) = 15.6$  Myr, which is older than that suggested by Pilbeam<sup>34</sup>. The preceding assumption may not hold well but is obviously better than the assumption of equal rate in both the rhesus monkey and orangutan lineages because the length (4.78) for the former lineage is considerably longer than that (2.98) for the latter. Therefore, this approach can substantially reduce the effect of unequal rates among lineages. Using this approach and assuming that owl monkey (the NW monkeys) branched off 35–45 Myr ago<sup>25–27</sup>, we estimate that the rhesus monkey (the OW monkeys) branchpoint is 24.0–30.9 Myr ago, the orangutan branchpoint is 15.0–19.3 Myr ago, the gorilla branchpoint is 7.7–9.9 Myr ago and the chimpanzee branchpoint is 7.4–9.5 Myr ago. These estimates are close to the dates proposed by Andrews<sup>35</sup> and Pilbeam<sup>25</sup>.

Instead of differences in generation time or, more precisely, in the number of germline DNA replications per year<sup>8,16</sup>, changes in DNA repair mechanisms<sup>9</sup> have recently been proposed as the primary cause of rate differences among mammals. We do not exclude the possibility that the DNA repair system is more effective in man than in rodents, but we do not find it necessary to invoke such changes to explain the large differences in substitution rate among mammals. The generation time in rodents (mouse and rat) is about 100 times shorter than that in man, so the number of germline DNA replications per year in rodents could be 10 times higher than that in man<sup>36</sup>. Such a large difference in the number of germline DNA replications per year would be sufficient to explain the approximately sevenfold difference in synonymous substitution rate between rodents

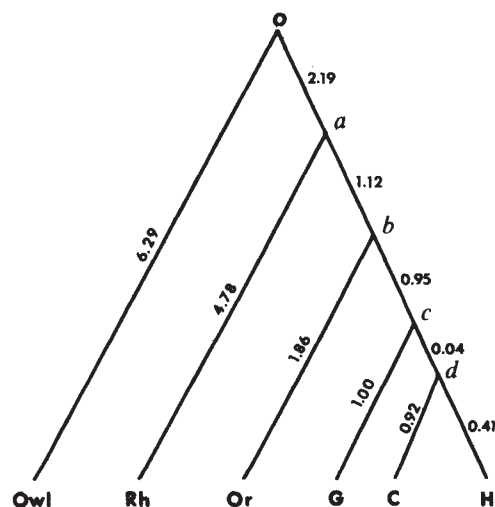


Fig. 1 A phylogenetic tree for the primates. The branching sequences were inferred by applying Li's method<sup>33</sup> to the distances between primate species in Table 3; the tree is also the maximum parsimony tree<sup>11</sup>. The number on each branch is the number of nucleotide substitutions per 100 sites. Using goat as a reference, we find that the distance from node *o* to rhesus monkey is  $38.20 - 37.52 = 0.68$  longer than that from node *o* to owl monkey (Table 3). Because the distance between owl monkey and rhesus monkey is 13.26, the distance from node *o* to owl monkey is  $(13.26 - 0.68)/2 = 6.29$  (Fig. 1) and that from node *o* to rhesus monkey is 6.97. Similarly, using owl monkey as a reference, we find that the distances from node *a* to rhesus monkey and orangutan are 4.78 and 2.98, respectively. Therefore, the distance from node *o* to node *a* is  $6.97 - 4.78 = 1.12$ . The distance from node *b* to orangutan was obtained using owl monkey and rhesus monkey as references and the other branch lengths were obtained using owl monkey, rhesus monkey and orangutan as references. Owl, Rh, Or, G, C and H denote owl monkey, rhesus monkey, orangutan, gorilla, chimpanzee and human, respectively.

and humans, on the assumption that the mutation rate per DNA replication is constant. Rhesus monkeys reproduce as early as age 3, whereas humans do not reproduce until age 12 or older. Again the difference in DNA replication number is potentially sufficient to account for the approximately twofold difference in rate of silent substitution between the rhesus monkey and human lineages. The generation time in artiodactyls is much closer to that in primates than to that in rodents, which is consistent with our observations on relative substitution rates.

The rate constancy assumption was an important argument in the formulation of the neutral mutation hypothesis<sup>37</sup> and has always been taken as strong support for it<sup>5</sup>. Violation of this assumption, however, should not be taken as evidence against the hypothesis, for a constant substitution rate would be predicted only if the mutation rate remains constant. Indeed, a serious criticism of the rate-constancy argument has been that the approximate constancy seen in protein sequence data is in terms of chronological time rather than generation number, but mutation rates in different organisms are more nearly comparable when measured in generations than in absolute time units. Therefore, the finding that the rate of nucleotide substitution is higher in short-living organisms, such as rodents, than in long-living organisms, such as humans, is actually more in line with the neutral mutation hypothesis than if the rates are equal for all organisms.

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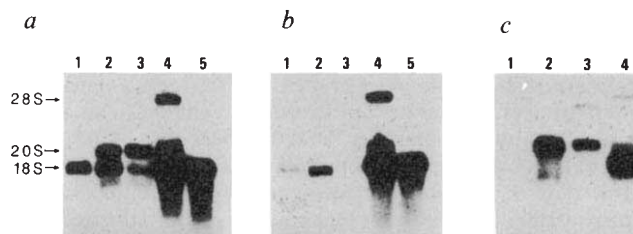
## Accelerated evolution in the reactive centre regions of serine protease inhibitors

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The serine protease inhibitors (serpins) are a family of proteins that function to control the action of serine proteases in many diverse physiological processes. The functional region or reactive centre of these inhibitors is near the C-terminal end and is an exposed site that acts as a bait for the appropriate serine protease to recognize and covalently bind. The specificity of the inhibitor is determined, at least in part, by a single amino acid that resides in this region at the P<sub>1</sub> position<sup>1</sup>. We show here that following a gene duplication event the reactive centres of three related rodent protease inhibitors have diverged from each other at unprecedented rates. This has resulted in proteins with different predicted specificities and we postulate that these changes were fixed by positive darwinian selection and that the most likely selective forces are extrinsic proteases, namely those used by parasites to facilitate their spread throughout the host.

We have isolated complementary DNA clones for several of the major rodent protease inhibitors that are synthesized in the liver and subsequently secreted into the plasma. One of these has been identified as the cDNA encoding contrapsin<sup>2</sup> which is most closely related to human  $\alpha_1$ -antichymotrypsin ( $\alpha_1$ -achy). Despite having a high degree of similarity, these two inhibitors have different protease specificities: contrapsin is an inhibitor of trypsin-like proteases<sup>3,4</sup>, whereas  $\alpha_1$ -achy is an inhibitor of chymotrypsin-like proteases<sup>5-7</sup>. The difference in specificity between these two proteins has been attributed to the dramatic degree of non-homology within the short stretch of amino acids encompassing the reactive centre region and particularly to the resulting change in the P<sub>1</sub> amino acid<sup>2</sup>. To explain this fundamental difference in protease specificity we have investigated these genes in rodents further. Although humans have only one  $\alpha_1$ -achy gene (unpublished data) we have shown that mouse and rat have multiple contrapsin-related genes<sup>8</sup>. In the mouse they are arranged in a tightly clustered array on chromosome



**Fig. 1** Northern blot analysis of mouse and rat liver poly(A)<sup>+</sup>RNA hybridized with different contrapsin cDNA probes. The probes used were *a*, the full length contrapsin cDNA; *b*, the 3' end of the contrapsin cDNA; *c*, a 16-nucleotide oligonucleotide. Hybridization conditions in *c* were 5×SSC at 37°C overnight and washed in 5×SSC at 42°C. Lanes 1-3, rat RNAs; lane 4 BALB/c mouse RNA and lane 5, C57BL/6 mouse RNA. RNA was isolated from rats which were (lane 1) normal, (lane 2) treated with bacterial lipopolysaccharides to induce an inflammatory reaction or (lane 3) treated with dexamethasone. RNA extractions<sup>24</sup> and Northern blot analysis<sup>25</sup> were performed as described.

12 designated the *Spi-2* locus. The possibility therefore arose that these related genes have evolved to acquire different functions, one of them equivalent to human  $\alpha_1$ -achy.

The messenger RNA isolated from mouse and rat liver contains at least two different contrapsin-related mRNA transcripts of ~1.8 kilobases (kb) (18S) and 2.2 kb (20S)<sup>8,9</sup>. (A third mRNA of 5 kb has not been well characterized.) The 2.2 kb mRNA is induced in the rat and in some mouse strains during an inflammatory response and also upon treatment with dexamethasone (Fig. 1*a* and ref. 9). We shall refer to these as '*spi-2*' mRNAs after the name of the genetic locus in the mouse<sup>8</sup>. The 1.8 kb mRNA species will be referred to as the *spi-2.1* mRNA in the rat, but as contrapsin mRNA in the mouse, where an activity has been established for the gene product<sup>2,3</sup>. The 2.2-kb mRNA will be referred to as the *spi-2.2* mRNA in both species.

In the rat, the *spi-2* RNAs are likely to be encoded by different genes (Fig. 1). Two DNA probes representing different regions of the contrapsin mRNA differentially hybridize to the rat *spi-2* mRNA species. A probe containing the 3' portion of the mouse contrapsin cDNA hybridizes specifically to the rat *spi-2.1* mRNA (Fig. 1*b*), whereas a synthetic oligonucleotide corresponding to a region in the middle of the mRNA hybridizes specifically to the rat *spi-2.2* mRNA (Fig. 1*c*).



**Fig. 2** Nucleotide sequence comparisons of rodent and human *spi-2* cDNAs. The sequences represent the 3' half of the mRNA. The contrapsin and human  $\alpha$ -achy sequences were previously reported<sup>5</sup>. All sequences are compared to mouse contrapsin cDNA, with only nucleotide changes shown in the other three sequences. The coding region sequences are divided into three regions by the degree of homology of the four sequences. Stop codons are boxed, and gaps are designated by dots. Sequences were determined by the chain termination method of Sanger<sup>26</sup>. Mouse contrapsin is designated here as con, the two rat *spi-2* genes are 2.1 (18S mRNA) and 2.2 (20S mRNA) and human  $\alpha_1$ -achy is  $\alpha$ -ac. The contrapsin 3' end probe (Fig. 1b) starts at nucleotide 510 and continues to the end; the 16-nucleotide oligomer (Fig. 1c) starts at nucleotide 7.

Region 1

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| 2.2          | A    | --A                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| $\alpha$ -ac | A    | --C                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 52           | con  | GAC                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 2.1          | A    | --T                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 2.2          | G    | ---                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| $\alpha$ -ac | ---  | ---                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 103          | con  | GTG                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 2.2          | ---  | --A                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 154          | con  | GAT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 205          | con  | AGC                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 2.1          | ATT  | ---                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 2.2          | ATG  | ---                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 358          | con  | CTG                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 2.1          | --A  | A-A                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 2.2          | --G  | ---                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 2.2          | ...  | AT-                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 613          | con  | GCT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 2.1          | AG-  | ---                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 2.2          | A--  | GCA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 664          | con  | GCT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| 2.2          | A    | --TC                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             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| $\alpha$ -ac | A    | --TG                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             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| 731          | con  | ACATCCTGTCAATCAAGCTGTGATTGGCT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    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| 2.2          | G    | --TT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             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                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | G--                                                                | C--      | ---  | ---  | --- | ---  | ---  | ---  | ---  | --- | --- | ---  | ---  | ---  | ---  | ---  | --- |
| 798          | con  | TCTCTGTGGTCAGT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | 2.1                                                                | A        | --G  | ---  | --- | ---  | ---  | ---  | ---  | --- | --- | ---  | ---  | ---  | ---  | ---  | --- |
| 2.2          | A    | --G                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| --- |
| $\alpha$ -ac | ---  | --G                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| --- |
| 865          | con  | TCTCTCTGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT |                                                                    |          |      |      |     |      |      |      |      |     |     |      |      |      |      |      |     |

**Table 1** Nucleotide sequence similarity and numbers of substitutions between *spi-2*-like genes

| Nucleotide sequence homology (%)                                                              |                       |                |                       |                |                                  |                |                         |                |                                    |                |                                    |                |
|-----------------------------------------------------------------------------------------------|-----------------------|----------------|-----------------------|----------------|----------------------------------|----------------|-------------------------|----------------|------------------------------------|----------------|------------------------------------|----------------|
| Region                                                                                        | con vs <i>spi-2.1</i> |                | con vs <i>spi-2.2</i> |                | <i>spi-2.1</i> vs <i>spi-2.2</i> |                | Con vs $\alpha_1$ -achy |                | <i>spi-2.1</i> vs $\alpha_1$ -achy |                | <i>spi-2.2</i> vs $\alpha_1$ -achy |                |
|                                                                                               |                       |                |                       |                |                                  |                |                         |                |                                    |                |                                    |                |
| 1                                                                                             | 84                    |                | 83                    |                | 84                               |                | 74                      |                | 75                                 |                | 77                                 |                |
| 2                                                                                             | 33                    |                | 28                    |                | 24                               |                | 41                      |                | 31                                 |                | 53                                 |                |
| 3                                                                                             | 75                    |                | 64                    |                | 65                               |                | 69                      |                | 60                                 |                | 57                                 |                |
| No. of substitutions per synonymous ( $K_S$ ) and non-synonymous ( $K_A$ ) site between genes |                       |                |                       |                |                                  |                |                         |                |                                    |                |                                    |                |
| Region                                                                                        | con vs <i>spi-2.1</i> |                | con vs <i>spi-2.2</i> |                | <i>spi-2.1</i> vs <i>spi-2.2</i> |                | con vs $\alpha_1$ -achy |                | <i>spi-2.1</i> vs $\alpha_1$ -achy |                | <i>spi-2.2</i> vs $\alpha_1$ -achy |                |
|                                                                                               | $K_S$                 | $K_A$          | $K_S$                 | $K_A$          | $K_S$                            | $K_A$          | $K_S$                   | $K_A$          | $K_S$                              | $K_A$          | $K_S$                              | $K_A$          |
| 1                                                                                             | 0.35<br>(0.07)        | 0.14<br>(0.02) | 0.38<br>(0.07)        | 0.16<br>(0.02) | 0.42<br>(0.08)                   | 0.13<br>(0.02) | 0.76<br>(0.12)          | 0.24<br>(0.03) | 0.69<br>(0.12)                     | 0.25<br>(0.03) | 0.70<br>(0.11)                     | 0.18<br>(0.02) |
| 2                                                                                             | NA                    | 1.38<br>(0.56) | NA                    | 1.42<br>(0.59) | 0.88<br>(0.86)                   | NA             | 2.24<br>(2.90)          | 0.99<br>(0.34) | 1.00<br>(0.59)                     | NA             | 0.79<br>(0.52)                     | 0.62<br>(0.23) |
| 3                                                                                             | 0.26<br>(0.15)        | 0.44<br>(0.11) | 1.33<br>(0.99)        | 0.39<br>(0.10) | 0.95<br>(0.51)                   | 0.50<br>(0.12) | 1.63<br>(0.99)          | 0.27<br>(0.08) | NA<br>(0.11)                       | 0.45<br>(0.11) | NA<br>(0.10)                       | 0.40<br>(0.10) |

Values in parenthesis are standard errors; NA, not applicable (no meaningful estimate can be obtained). Abbreviations for cDNAs are as follows: con, mouse contrapsin gene;  $\alpha_1$ -achy, human  $\alpha_1$ -anti-chymotrypsin gene; *spi-2.1* and *spi-2.2*, rat *spi-2* mRNAs (see text).

The  $K_S$  values should more closely reflect the rate of divergence of two genes.

Table 1 also shows  $K_S$  and  $K_A$  values for each of the six possible comparisons for the *spi-2* like genes subdivided into the three regions (W.-H. Li kindly carried out this analysis). From these values we draw the following conclusions. First, in region 1 as in all protein-coding regions analysed so far, the  $K_S$  value is several times greater than the  $K_A$  value. The average ratio of  $K_S/K_A$  for all possible comparisons of region 1 in the rodent protease inhibitor genes is 2.7. The average ratio of  $K_S/K_A$  for 37 mammalian genes analysed by this method is 5.3. However, in the more rapidly evolving proteins such as interferon- $\alpha_2$ ,  $\beta$ -globin and relaxin this ratio is between 2 and 3. Therefore serine protease inhibitors in region 1 compare with other known proteins; albeit the proteins that evolve most rapidly. Secondly,  $K_A$  for region 2 equals or exceeds the  $K_S$  value of region 1. In some comparisons (mouse contrapsin gene versus either rodent gene), the  $K_A$  values for region 2 are four times greater than the  $K_S$  values for region 1. This high degree of substitution in region 2 indicates that the part of the gene containing the reactive centre has undergone accelerated evolution; a process postulated to occur in genes following a duplication event<sup>11-13</sup>. Note that the values for region 2, because of the high degree of divergence, are in some cases not calculable. Where they are calculable, conservative estimates are stated: the actual values could be much greater. Finally, the  $K_A$  values in region 3, although lower than in region 2, reveal that this segment of the genes is also evolving at an accelerated rate, because the numbers of substitutions in all but one comparison (contrapsin versus  $\alpha_1$ -achy) are appreciably higher than the  $K_A$  values for region 1 and, in the rodent comparisons at least, as high as the  $K_S$  values of region 1.

To determine the rate at which these genes have evolved, and to allow comparisons with rates for other genes studied, the evolutionary history of this gene family must be established. We suggest, that the genes that code for the *spi-2* mRNAs are a result of a duplication event that occurred before mouse and rat divergence and also that the mouse contrapsin and rat *spi-2.1* genes are orthologous as are the mouse and rat *spi-2.2* genes. These conclusions are drawn for the following reasons. (1) Messenger RNAs of 18S (*spi-2.1*) and 20S (*spi-2.2*) are found in both species (Fig. 1); (2) there is a high constitutive level of the *spi-2.1* mRNA in both species; (3) the *spi-2.2* mRNA is induced by inflammation and dexamethasone in at least some strains of mice<sup>8</sup> and in rats (Fig. 1); (4) a 3'-end DNA probe specific for the rat *spi-2.2* mRNA hybridizes only to the mouse *spi-2.2* mRNA (data not shown); a similar probe specific for mouse contrapsin hybridizes only to rat *spi-2.1* (Fig. 1b). This is supported by the degree of nucleotide similarity for the *spi-2.1*

genes in region 3 (Table 1, top); (5) attempts to detect other *spi-2*-like mRNA species in mouse or rat and to isolate another class of related cDNA clones from rat-liver libraries have failed. By definition, the orthologous genes have been separate only since mouse and rat divergence. The date of this divergence is controversial and estimates range from 10 to 30 Myr ago, although 15 Myr is considered a reasonable estimate (W.-H. Li, personal communication). To determine when the gene duplication event occurred, we have taken region 1 as representing that part of the gene evolving comparably to other rodent genes analysed. Comparing the  $K_S$  value for region 1 of the rat *spi-2.1* and *spi-2.2* genes to the  $K_S$  value of the putative orthologous genes, mouse contrapsin and rat *spi-2.1*, we predict that the duplication occurred some 3 Myr before mouse-rat divergence. The rate of divergence of two orthologous genes is defined as the number of substitutions occurring per site per year; a value of  $46 \times 10^{-9}$  is obtained for the nonsynonymous sites in region 2 of the mouse contrapsin and rat *spi-2.1* comparison ( $23-69 \times 10^{-9}$  taking the range of dates of 10-30 Myr). Analysis of many genes has shown that the most rapidly evolving bases are those in synonymous positions of codons, 3' untranslated regions, 3' flanking regions of genes and pseudogenes<sup>14</sup>. Pseudogenes evolve most rapidly, although the differences in rates amongst the above categories is on average <30%. Pseudogenes are believed to be subject to no functional constraints and should accumulate substitutions at a rate that reflects the mutation rate. When 14 different orthologous pairs of genes were compared between mouse and rat the average rate of substitution at synonymous sites was found to be  $8 \times 10^{-9}$  per base per year (W.-H. Li, personal communication). For two mouse pseudogenes ( $\psi\alpha 3$  globin and immunoglobulin VH108B) the rate of divergence was  $6 \times 10^{-9}$  per base per year<sup>15</sup>. Thus in region 2 the rate of substitution at nonsynonymous positions is 6-8 times greater than that for the most rapidly evolving stretches of DNA.

Considering the unlikely event that rat *spi-2.2* and contrapsin are orthologous or indeed none of the pairs of genes we have compared is orthologous, the conclusion of an accelerated rate of divergence in the reactive centre region is still inescapable. If we further postulate that region 2 is actually a non-functional region of the protein and therefore is accumulating substitutions at an unconstrained rate (at the rate of pseudogenes) then these genes would have become separated some 100-150 Myr ago: unlikely because regions 1 of the human and rodent inhibitor genes are diverging at close to the rate commonly found for a number of genes.

The substitutions in the reactive-centre regions of these proteins have resulted in inhibitors with varying specificities. Aligning the reactive centres of the two rat genes, *spi-2.1* and *spi-2.2*, to the human sequence in which the P<sub>1</sub> position is known<sup>16</sup>



|              |     |          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
|--------------|-----|----------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|--|
|              | 1   |          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| con          | V   | V        | L | V | N | Y | I | Y | F | K | G | K | W | K | I | S | F | D | P | Q |  |
| 2.1          | M   | V        | L | V | N | Y | I | Y | F | K | G | K | W | K | V | P | F | N | P | R |  |
| 2.2          | M   | V        | L | V | N | Y | I | Y | F | K | G | K | W | K | V | P | F | D | P | R |  |
| $\alpha$ -ac | M   | V        | L | V | N | Y | I | F | F | K | A | K | W | E | M | P | F | D | P | Q |  |
|              | 21  |          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| con          | D   | T        | F | E | S | E | F | Y | L | D | E | K | R | S | V | K | V | P | M | M |  |
| 2.1          | D   | T        | F | E | S | E | F | Y | L | D | E | K | R | S | G | K | V | P | M | M |  |
| 2.2          | D   | T        | F | Q | S | E | F | Y | S | G | K | R | R | P | V | K | V | P | M | M |  |
| $\alpha$ -ac | D   | T        | H | Q | S | R | F | Y | L | S | K | K | K | W | V | M | V | P | M | M |  |
|              | 41  |          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| con          | K   | M        | K | L | L | T | T | R | H | F | R | D | E | E | L | S | C | S | V | L |  |
| 2.1          | K   | I        | K | E | V | T | T | P | Y | V | R | D | E | E | L | S | C | S | T | V |  |
| 2.2          | K   | L        | E | D | L | T | T | P | Y | V | R | D | E | E | L | N | C | T | V | V |  |
| $\alpha$ -ac | S   | L        | H | H | L | T | I | P | Y | F | R | D | E | E | L | S | C | T | V | V |  |
|              | 61  |          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| con          | E   | L        | K | Y | T | G | N | A | S | A | L | L | I | L | P | D | Q | G | R | M |  |
| 2.1          | E   | L        | K | Y | T | G | N | A | S | A | L | F | I | L | P | D | Q | G | K | M |  |
| 2.2          | E   | L        | K | Y | T | G | N | A | S | A | L | F | I | L | P | D | Q | G | K | M |  |
| $\alpha$ -ac | E   | L        | K | Y | T | G | N | A | S | A | L | F | I | L | P | D | Q | D | K | M |  |
|              | 81  |          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| con          | Q   | Q        | V | E | A | S | L | Q | P | E | T | L | R | K | W | R | K | T | L | F |  |
| 2.1          | Q   | Q        | V | E | S | S | L | Q | P | E | T | L | R | K | W | R | K | D | S | L |  |
| 2.2          | Q   | Q        | V | E | A | S | L | Q | P | E | T | L | R | K | W | R | K | D | S | L |  |
| $\alpha$ -ac | E   | E        | V | E | A | M | L | L | P | E | T | L | K | R | W | R | D | S | L | E |  |
|              | 101 |          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| con          | P   | S        | Q | I | E | E | L | N | L | P | K | F | S | I | A | S | N | Y | R | L |  |
| 2.1          | P   | R        | I | I | N | D | L | R | M | P | K | F | S | I | S | T | A | D | Y | S |  |
| 2.2          | P   | S        | M | I | T | D | E | L | Y | L | P | K | F | S | I | S | A | D | Y | N |  |
| $\alpha$ -ac | F   | R        | E | I | G | E | L | Y | L | P | K | F | S | I | S | R | D | Y | N | L |  |
|              | 121 |          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| con          | E   | E        | D | V | L | P | E | M | G | I | K | E | V | F | T | E | Q | A | D | L |  |
| 2.1          | -   | K        | E | V | L | P | E | L | G | I | K | K | V | F | S | Q | T | A | D | L |  |
| 2.2          | -   | E        | D | V | L | P | E | L | G | I | K | E | V | F | S | Q | Q | A | D | L |  |
| $\alpha$ -ac | -   | N        | D | I | L | L | Q | L | G | I | E | E | A | F | T | S | K | A | D | L |  |
|              | 141 |          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| con          | S   | G        | I | I | E | T | K | K | L | S | V | S | Q | V | V | H | K | A | V | L |  |
| 2.1          | S   | R        | I | T | G | T | K | D | L | Y | V | S | Q | V | V | H | K | A | V | L |  |
| 2.2          | S   | G        | I | T | G | D | K | D | L | M | V | S | E | V | V | H | K | A | V | L |  |
| $\alpha$ -ac | S   | G        | I | T | G | A | R | N | L | A | V | S | Q | V | V | H | K | A | V | L |  |
|              | 161 |          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| con          | D   | V        | A | E | T | G | T | E | A | A | A | A | T | G | V | I | G | G | I | R |  |
| 2.1          | D   | V        | D | E | T | G | T | E | A | T | A | A | T | G | V | A | T | V | I | R |  |
| 2.2          | D   | V        | A | E | T | G | T | E | A | A | A | A | T | G | V | K | F | V | P | V |  |
| $\alpha$ -ac | D   | V        | F | E | E | G | T | E | A | S | A | A | T | A | V | K | I | T | L | L |  |
|              | 181 | Region 2 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| con          | K   | A        | I | L | P | - | - | - | A | V | H | F | N | R | P | F | L | F | V | I |  |
| 2.1          | R   | Q        | P | R | T | - | - | - | L | N | F | N | R | P | F | F | M | V | F | I |  |
| 2.2          | S   | A        | K | L | D | P | L | - | I | A | F | F | N | R | P | F | L | M | I | I |  |
| $\alpha$ -ac | S   | A        | L | V | E | T | R | T | I | V | R | F | N | R | P | F | L | M | I | I |  |
|              | 201 |          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |
| con          | Y   | H        | T | S | A | Q | S | I | L | F | M | A | K | V | N | N | P | K | ! | ! |  |
| 2.1          | T   | D        | M | D | S | Q | S | I | L | F | V | A | K | I | T | M | P | S | ! | ! |  |
| 2.2          | S   | D        | T | E | T | A | I | A | P | F | L | A | K | I | F | T | N | P | K | ! |  |
| $\alpha$ -ac | V   | P        | T | D | T | Q | N | T | F | F | M | S | K | V | T | N | P | K | Q | A |  |

**Fig. 3** Comparison of the predicted protein sequences for all four *spi-2* genes. Amino acids the same for three of the four proteins are boxed. Only region 2 is designated. Amino acids are shown in single-letter code. The  $P_1$  amino-acid position is indicated by a dot.

allows predictions of the inhibitory activities. The  $P_1$  amino acid predicted in *spi-2.1* is an arginine whereas in *spi-2.2* a valine lies at this position. A positively charged amino acid at this position usually results in an inhibitor with anti-tryptic activity<sup>17</sup>, whereas a valine results in anti-elastolytic activity<sup>18,19</sup>. The mouse contrapsin, like the rat *spi-2.1* protein, contains a positively charged amino acid at the  $P_1$  position. It remains to be determined how the amino acids in the surrounding area affect the physiological function of these inhibitors. However, it has been shown by X-ray crystallography of a model system<sup>20</sup> that the four amino acids N-terminal to the  $P_1$  site are in direct contact with the complexed protease.

How can we account for the high rate of substitution in the reactive centre region of these proteins? We postulate that in region 2 of the protein, nucleotide substitutions are being rapidly fixed by positive darwinian selection. Changes that advantageously affect the activity of the protein would be fixed more rapidly in a population than would neutral mutations. It is unlikely that these inhibitors are acting exclusively, or at all, upon indigenous proteases as this would require rapid co-evolution of the target protease. We feel it is more likely that these inhibitors are acting upon exogenous proteases brought in by infectious agents upon wounding or parasitic infection. It is known that parasites such as *Schistosoma mansoni*<sup>21</sup>, and bacteria such as *Pseudomonas*<sup>22</sup> synthesize serine proteases that act as virulence factors to increase the efficiency of infection. There-

fore, inhibition of these types of proteases would provide an appreciable selective advantage to the host. However, it is unlikely that all of the substitutions that have arisen in the *spi-2* genes have been selected for. We suggest that many of the mutations have been carried along with the advantageous substitutions by the process called 'hitchhiking'<sup>23</sup>. This may explain the overall rapid divergence (low  $K_S/K_A$  ratio) of these proteins. The molecular mechanism by which the rapid rate of divergence in region 2 has occurred may be elucidated by the study of more closely related rodent species.

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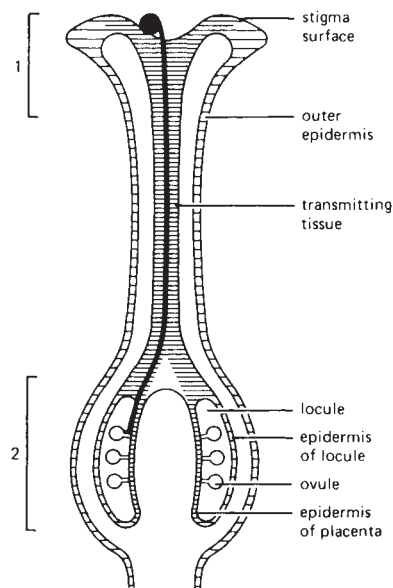
## Developmentally controlled expression of a gene associated with self-incompatibility in *Nicotiana glauca*

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In many flowering plant species, a system of self-incompatibility, typically controlled by a single gene with multiple alleles (*S*-gene)<sup>1</sup>, enables individual plants to recognize and reject their own pollen. In gametophytically determined systems, the growth of self-pollen tubes is arrested in the style or occasionally the ovary. The expression of this self-incompatibility is developmentally regulated, being strongest in mature flowers and weak in immature styles<sup>1,2</sup>. We have used a complementary DNA clone, believed to encode the *S*<sub>2</sub>-allele of *Nicotiana glauca*<sup>2,3</sup>, to detect *S*<sub>2</sub> messenger RNA by *in situ* hybridization to sections. We report here that expression of the gene occurs in the stigma and throughout the secretory tissue, but not in other parts, of mature pistils. In



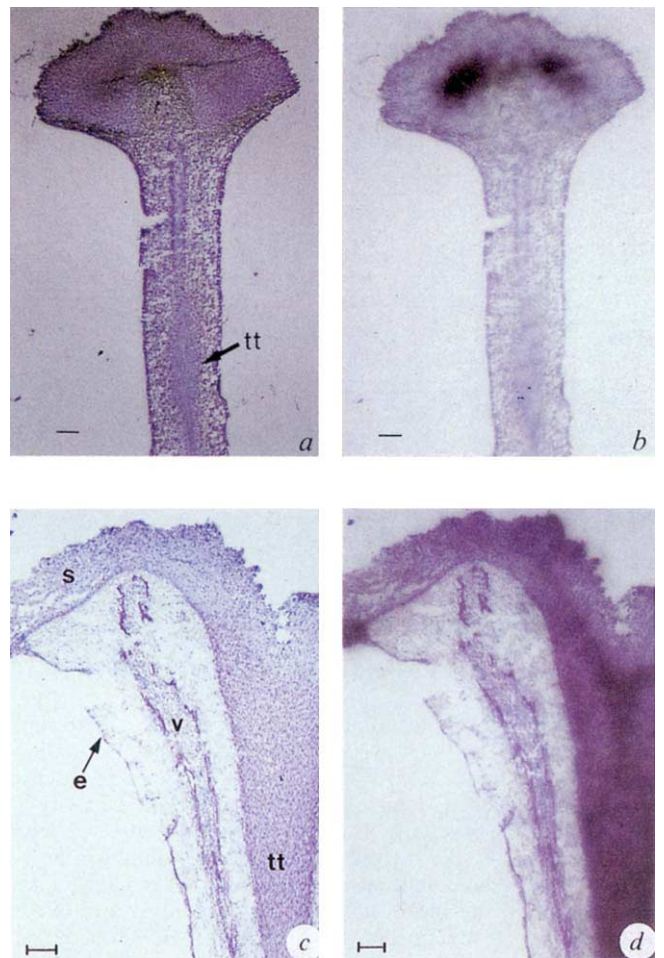
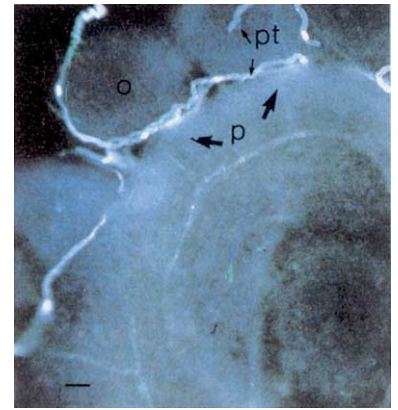
**Fig. 1** Diagram of compatible pollination of a mature pistil of *Nicotiana alata*. The secretory epidermis of the stigma, transmitting tract of the style and the epidermis of the placenta are indicated by close cross-hatching; these are the tissues which hybridize with  $S_2$  cDNA and correspond to the path taken by the pollen tube before entering the ovule. The outer epidermis of the pistil and the epidermis of the locule originate from the same cell line as the transmitting tissues and are indicated by wide cross-hatching. Area 1, sections through the stigma and style as in Fig. 3. Area 2, sections through this ovary region as in Figs 2 and 4.

**immature flowers,  $S_2$  mRNA is confined to the proliferated epidermis of the stigma. We conclude that  $S_2$ -gene expression correlates well with the expression of incompatibility.**

During maturation of pistils of the Solanaceae, the epidermal layer (layer LI, ref. 4) initially divides to form the epidermis of the placenta and the ovary locule as well as the initials of the stigma surface secretory tissue and the transmitting tissue. This is followed by cell division to form the receptive stigma, and cell division and elongation to form the transmitting tissue of the style. Development of this tissue proceeds from the top to the base of the style<sup>4</sup>. Thus, there is a continuous secretory tissue connecting the stigma surface, the style transmitting tract and the inner epidermis of the ovary (which includes both the epidermis of the placenta and the outer epidermis in each locule) (Fig. 1). During a compatible pollination, pollen received on the stigma surface germinates to produce pollen tubes which grow extracellularly through the transmitting tissue of the style, then over the surface of the placental epidermis (Fig. 2) into the micropyles of the ovules. The sperm cells are then released from the tube tip to effect the double fertilization characteristic of flowering plants. In this paper, we report the use of hybridization histochemistry to show the development of tissue-specific expression of the gene corresponding to  $S_2$  cDNA (designated the  $S_2$ -gene) of *N. alata*.

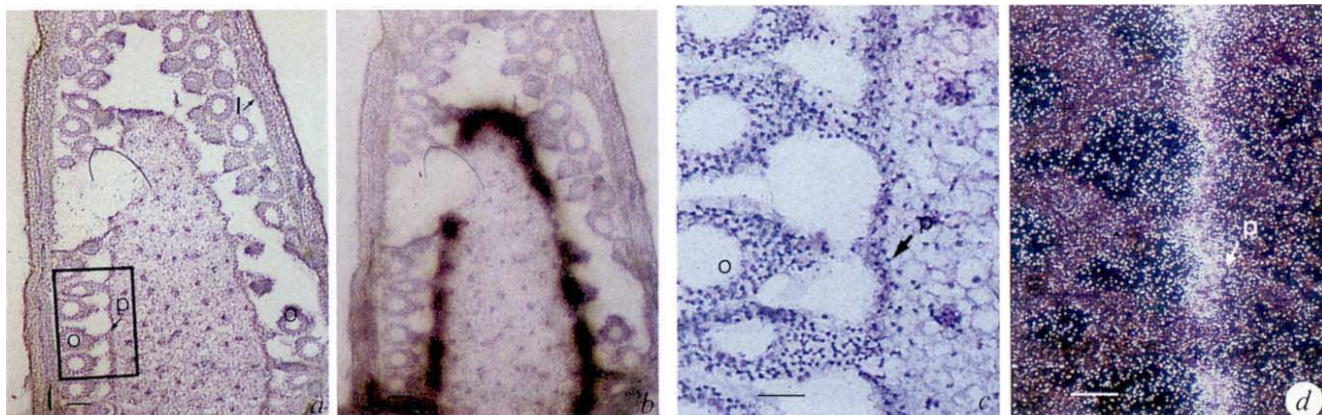
We reported previously that we were unable to detect hybridization of the  $S_2$  cDNA to mRNA isolated from immature styles using Northern analysis<sup>2</sup>. More recently, we have shown by primer extension experiments, that the immature pistil has very low levels of  $S_2$  mRNA (M. A. Anderson, unpublished data). Hybridization of the  $S_2$  cDNA to longitudinal sections of immature pistils (Fig. 3) showed that hybridization was restricted to two well-defined sites in the stigma which correspond to the proliferated stigma epidermis. There was no detectable hybridization to the transmitting tract or ovary of immature pistils. In *N. alata* ( $S_2S_3$ ), self pollen tubes are inhibited after growing through the upper third of the style. Thus, pollen-tube arrest does not occur immediately after initial contact with the female

**Fig. 2** Fluorescence micrograph of a hand section through the ovary stained with aniline blue fluorochrome (Biosupplies Australia) showing pollen tubes (pt) growing over the surface of the placenta (p) toward the ovules (o). Bar, 50  $\mu$ m.



**Fig. 3** *a, b*, Hybridization of  $S_2$ -cDNA to a longitudinal section of an immature pistil of *Nicotiana alata*. *a*, Longitudinal cryostat section through immature pistil; *b*, composite image produced with autoradiograph of same section after hybridization of  $^{32}$ P-labelled  $S_2$ -cDNA. Binding is restricted to two sites in the secretory epidermis of the stigma. The transmitting tract (tt) is differentiated but does not bind the  $S_2$ -cDNA. Bar, 100  $\mu$ m. *c, d*, Hybridization of  $S_2$ -cDNA to a longitudinal section of a mature pistil of *Nicotiana alata*. *c*, Longitudinal cryostat section of upper 2.5 mm of mature pistil corresponding to area 1 in Fig. 1, showing the structural continuity between the secretory tissue of the stigma (s) and the transmitting tissue (tt) of the style; v, vascular bundle. *d*, Composite image produced with autoradiograph of the same section after hybridization of  $^{32}$ P-labelled  $S_2$ -cDNA, showing localization of  $S_2$ -mRNA restricted to secretory tissue of the stigma and transmitting tract. There is no binding to the outer epidermis (e) of the style, the vascular bundle or the cortical parenchyma. Bar, 100  $\mu$ m.





**Fig. 4** Hybridization of  $S_2$  cDNA to a longitudinal section of a mature ovary. *a*, Longitudinal cryostat section of mature ovary, corresponding to area 2 of Fig. 1. The epidermis of the placenta (p), the epidermis of the locule (l) and the ovules (o) are indicated. The section is damaged and does not show the complete ovary; this is usual with cryostat sections of organs such as ovary which contain large, fluid-filled cavities. Bar, 100  $\mu$ m. *b*, Composite image produced with autoradiograph of same section as *a* after hybridization of  $^{32}$ P-labelled  $S_2$  cDNA, showing localization in epidermis of the placenta. *c*, *d*, Section from the boxed area of *a* after hybridization and liquid emulsion autoradiography. *c*, Bright-field micrograph showing four ovules (o) attached to the placenta. The epidermis of the placenta (p) is indicated. Bar, 50  $\mu$ m. *d*, The same section as *c* photographed with dark-field illumination. The white area over the epidermis of the placenta (p) corresponds to silver particles produced from the action of the bound  $^{32}$ P-cDNA on the photographic emulsion. The use of liquid emulsion enhances the resolution of the technique, enabling localization to particular cell layers.

**Methods.** Flowers of *Nicotiana glauca* of self-incompatibility genotypes  $S_2S_3$  were obtained as described<sup>2</sup>. Pistils were excised and immersed in OCT compound (Lab-Tek Products) in an embedding mould and frozen in Freon cooled with liquid nitrogen. Sections (6–8  $\mu$ m) were cut on a Reichert Jung cryostat at  $-12$  to  $-15^\circ\text{C}$ . The sections were transferred to slides pre-coated with 1% gelatine hardened with 0.25% formaldehyde by pressing the slide lightly against the section. Slides were transferred to a block of dry ice for  $\geq 30$  min to enhance adhesion of section to slide<sup>9</sup>. Control sections were incubated with ribonuclease A (Boehringer-Mannheim; 0.5 mg ml<sup>-1</sup>, 37  $^\circ\text{C}$ , 2 h) and washed. The sections were lightly fixed (2% glutaraldehyde, 0.15 M sodium phosphate buffer, pH 7.2, 33% ethylene glycol, 4  $^\circ\text{C}$ , 5–10 min), washed briefly in hybridization buffer (5 $\times$  Denhardt's, 50 mM sodium-phosphate pH 7.0, 600 mM NaCl, 5 mM EDTA, 1 mg ml<sup>-1</sup> sonicated herring sperm DNA and 50% deionized formamide), first at room temperature and then at 38  $^\circ\text{C}$ . Sections were pre-hybridized with hybridization buffer; 30 min, 38  $^\circ\text{C}$  and washed sequentially in 1 $\times$  SSC and ethanol (AR grade). For hybridization, labelled probe (6 ng DNA,  $6 \times 10^5$  c.p.m.) was applied in a volume large enough to cover the entire section (usually about 3  $\mu$ l). The section was covered with a coverslip and a sheet of parafilm, stored in an atmosphere of 50% formamide and incubated for 30 h at 40  $^\circ\text{C}$ . Sections were then washed sequentially in 4 $\times$  SSC at room temperature, 2 $\times$  SSC at room temperature, 1 $\times$  SSC for 30 min at 38  $^\circ\text{C}$  with gentle agitation and finally in ethanol (AR grade). Slides were exposed directly to X-ray film (Cronex MRF 32, Dupont). The autoradiograph was overlaid on the stained section to give the composite shown. Alternatively, slides were subjected to liquid emulsion autoradiography (Ilford Nuclear Research Emulsion)<sup>9</sup>. Slides were counterstained with toluidine blue (0.025%) to show the tissue structure. Probes of cDNA were labelled to a specific activity of  $10^8$  c.p.m. g<sup>-1</sup> using random priming<sup>10</sup>. Control experiments demonstrated that the hybridization observed represents specific binding to RNA. To demonstrate that RNA was preserved, pre-hybridized sections were stained for nucleic acid with acridine orange<sup>11</sup>. There was intense orange staining in all tissue types, but pre-incubation of replicate sections with ribonuclease A completely abolished the orange staining. RNA preservation was also demonstrated using a cDNA probe for ribosomal RNA. This probe, a 2.6-kilobase *Bam*HI fragment purified<sup>12</sup> from a *Bam*HI digestion of the pea ribosomal DNA plasmid pHA2 (from Dr J. Watson, Carnegie Institution, California), hybridized throughout the pistil tissues. As a negative control, we incubated sections with a cDNA encoding the enzyme (1 $\rightarrow$ 3) $\times$ (1 $\rightarrow$ 4)- $\beta$ -glucan hydrolase (EC 3.2.1.73) from barley<sup>13</sup>. This enzyme specifically hydrolyses the (1 $\rightarrow$ 3)(1 $\rightarrow$ 4)-glucan characteristic of cereals and grasses. Neither the mixed linked glucan nor the enzyme have been found in dicotyledons<sup>14</sup>. There was no binding of the (1 $\rightarrow$ 3)(1 $\rightarrow$ 4)- $\beta$ -glucan hydrolase cDNA to any sections.

tissue expressing the *S*-gene product. We suggest that the low level of expression restricted to the stigma in the immature flower is insufficient to induce pollen-tube arrest.

Hybridization to sections of the mature pistil (Fig. 3) showed that the  $S_2$ -gene was expressed throughout the transmitting tract of the style and also in the continuation of this tissue in the stigma. The expression of this gene, initially at a low level in the immature style and then at a high level in the mature style, is consistent with a number of earlier observations of developmentally regulated expression of self-incompatibility associated style proteins<sup>3,5</sup>. There was no hybridization to the outer epidermal or cortical cells of the mature style. The gene is expressed in the ovary of the mature flower but only in the epidermal secretory cells of the placenta (Fig. 4). As these cells are continuous with and have the same origin as those of the transmitting tract, hybridization of the  $S_2$  cDNA is not unexpected. That the hybridization does not extend into the epidermis of the carpel wall (the outer epidermis of the locule) and of the ovary is probably connected to the early, independent development of the placenta in relation to the other carpel tissues<sup>6</sup>. The pattern of  $S_2$ -gene expression, first in the immature stigma but not in the placenta or the transmitting tissue of the style, and later in all three, reflects the developmental sequence of the continuous secretory tissue<sup>4</sup>. *S*-gene expression in the mature style coincides precisely with the pathway that compatible pollen tubes take during their growth to the ovary (Figs 1 and 2). *S*-gene

expression in the placenta may have a physiological function in arresting growth of any self-pollen tubes which escape the normal inhibition zone. It might also represent an ancestral functional condition in the carpel; the properties of the placental epidermis being transferred, according to Corner's principle<sup>7</sup>, to the transmitting tract of the style with the evolution of the terminal stigma. In certain species with hollow styles or without styles, gametophytic self-incompatibility is characterized by pollen-tube inhibition within the ovary. The present observations give a molecular basis for the interpretation<sup>8</sup> of ovarian incompatibility as delayed expression of a normal gametophytically controlled *S*-gene.

This study illustrates the power of hybridization histochemistry in localizing gene expression in plant tissues and cell types; it will be valuable for analysing *S*-gene expression in pollen, which to date has eluded biochemical analysis.

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**Note added in proof:** Since this paper was submitted we have raised an antibody against a synthetic peptide corresponding to amino acids 45–66<sup>2</sup> of the  $S_2$  glycoprotein. Immunofluorescence microscopy with this antibody has shown that the  $S_2$  mRNA is translated both in the style transmitting tract and the epidermal cells of the placenta of the mature *N. glauca* pistil.

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## Sequences of *S*-glycoproteins, products of the *Brassica campestris* self-incompatibility locus

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The cruciferous genus *Brassica* has a self-incompatibility system that is under the sporophytic control of a single locus, *S* (ref. 1). Although evidence has accumulated that *S*-locus-specific proteins, termed *S*-glycoproteins<sup>2–4</sup>, are strongly associated with the expression of self-incompatibility<sup>4–8</sup>, their structures and functions are still unknown. This paper reports the sequences of the *S*-glycoproteins from three homozygotes of *Brassica campestris*. They show extensive homology. The published DNA sequence data for the *S*<sub>6</sub>-glycoprotein of *B. oleracea*<sup>9</sup> can be revised to encode the total sequence of the glycoprotein on the basis of the sequences of *S*-glycoproteins of *B. campestris*. These *S*-glycoproteins have cysteine-rich clusters and 6–7 oligosaccharide chains. There is no homology between the *S*-glycoproteins of *Nicotiana glauca*<sup>10</sup> and those of *B. campestris*.

In our previous papers<sup>11,12</sup>, we reported the isolation of the *S*-glycoproteins of three homozygotes, *S*<sub>8</sub>*S*<sub>8</sub>, *S*<sub>9</sub>*S*<sub>9</sub> and *S*<sub>12</sub>*S*<sub>12</sub> of *B. campestris* and their amino-terminal sequences. We also reported structures of the major *N*-glycosidic oligosaccharide chains of the total stigma glycoproteins of the homozygous genotype *S*<sub>8</sub>*S*<sub>8</sub> and presented evidence that these may be identical with the structures of the oligosaccharide chains of the *S*-glycoproteins of *B. campestris*.

Peptide fragments from *S*<sub>8</sub>-glycoprotein were obtained by cyanogen bromide fragmentation and digestion of the isolated glycoprotein with proteolytic enzymes. Sequence analysis of the intact glycoprotein and the peptide fragments revealed five partial sequences (Fig. 1a). The relative molecular mass (*M*<sub>r</sub>) of the protein part of *S*<sub>8</sub>-glycoprotein was calculated to be ~47,000 (47K) because *S*<sub>8</sub>-glycoprotein exhibited a broad band of *M*<sub>r</sub> 53K on SDS-polyacrylamide gel electrophoresis<sup>11</sup> and should contain four or five *N*-glycosidic carbohydrate chains<sup>12</sup>. Therefore the sequences in Fig. 1a should cover ~95% of the whole protein sequence.

The HPLC pattern of lysyl endopeptidase digests of the *S*<sub>8</sub>-glycoprotein (Fig. 1b), was quite different from those of *S*<sub>9</sub>- and *S*<sub>12</sub>-glycoproteins. The sequencing of these fragment peptides of *S*<sub>9</sub>- and *S*<sub>12</sub>-glycoproteins revealed many peptide fragments homologous with those of *S*<sub>8</sub>-glycoprotein (Fig. 1a). These results indicate that although displacement of amino acids was observed at many places, these *S*-glycoproteins are similar to each other over the whole region. This is consistent with the

hypothesis that these are products of alleles of a single locus. The underlined sequences in fragment C in Fig. 1a have parts that differ between the established sequences.

Several X-(a.a.)-Ser/Thr sequences were found, where X is a *N*-glycosylated asparaginyl residue of the type commonly observed in glycoproteins<sup>13</sup> and a.a. is any amino acid. Six such sites were found in the fragments of *S*<sub>8</sub>-glycoprotein. Note that amino acid 35 in fragment C of *S*<sub>8</sub>-glycoprotein, which is probably also a glycosylated asparagine, is replaced by aspartic acid in *S*<sub>12</sub>-glycoprotein. The fact that no galactose or arabinose was detected in the acid hydrolysate of the *S*<sub>8</sub>-glycoprotein (data not shown) shows that there may be no *O*-glycosidic saccharide chains in the *S*-glycoproteins of *B. campestris*.

Recently Nasrallah *et al.* reported a complementary DNA sequence encoding part of the *S*<sub>6</sub>-locus-specific glycoprotein (*S*<sub>6</sub>-glycoprotein)<sup>9</sup> in *B. oleracea*, a species very closely related to *B. campestris*. Their proposed nucleotide sequence of *S*<sub>6</sub>-glycoprotein (Fig. 2) is composed of 1,283 base pairs (bp). As there is a nonsense codon (TGA) at base pair positions 370–372 in the reading frame, they concluded that the nucleotides encode 123 amino-acid residues of the carboxy-terminal sequence of *S*<sub>6</sub>-glycoprotein. But a comparison of our peptide sequences of *S*<sub>8</sub>-, *S*<sub>9</sub>- and *S*<sub>12</sub>-glycoproteins with their cDNA sequence of *S*<sub>6</sub>-glycoprotein reveals the following. (1) A peptide sequence homologous to our amino-terminal sequences (fragment A) is found at their proposed carboxy-terminal region (from position 40). (2) The sequences homologous with fragments D and E can be observed in the downstream region of the termination codon, from positions 931 and 1,042, respectively, which was previously thought to be an unusual 3'-noncoding region. (3) Fragment C can be arranged on their sequence as a homologous sequence starting from position 292. The homology suddenly breaks after Arg at position 328, but if one base pair could be inserted between 330 and 337, the homology with their sequence would continue.

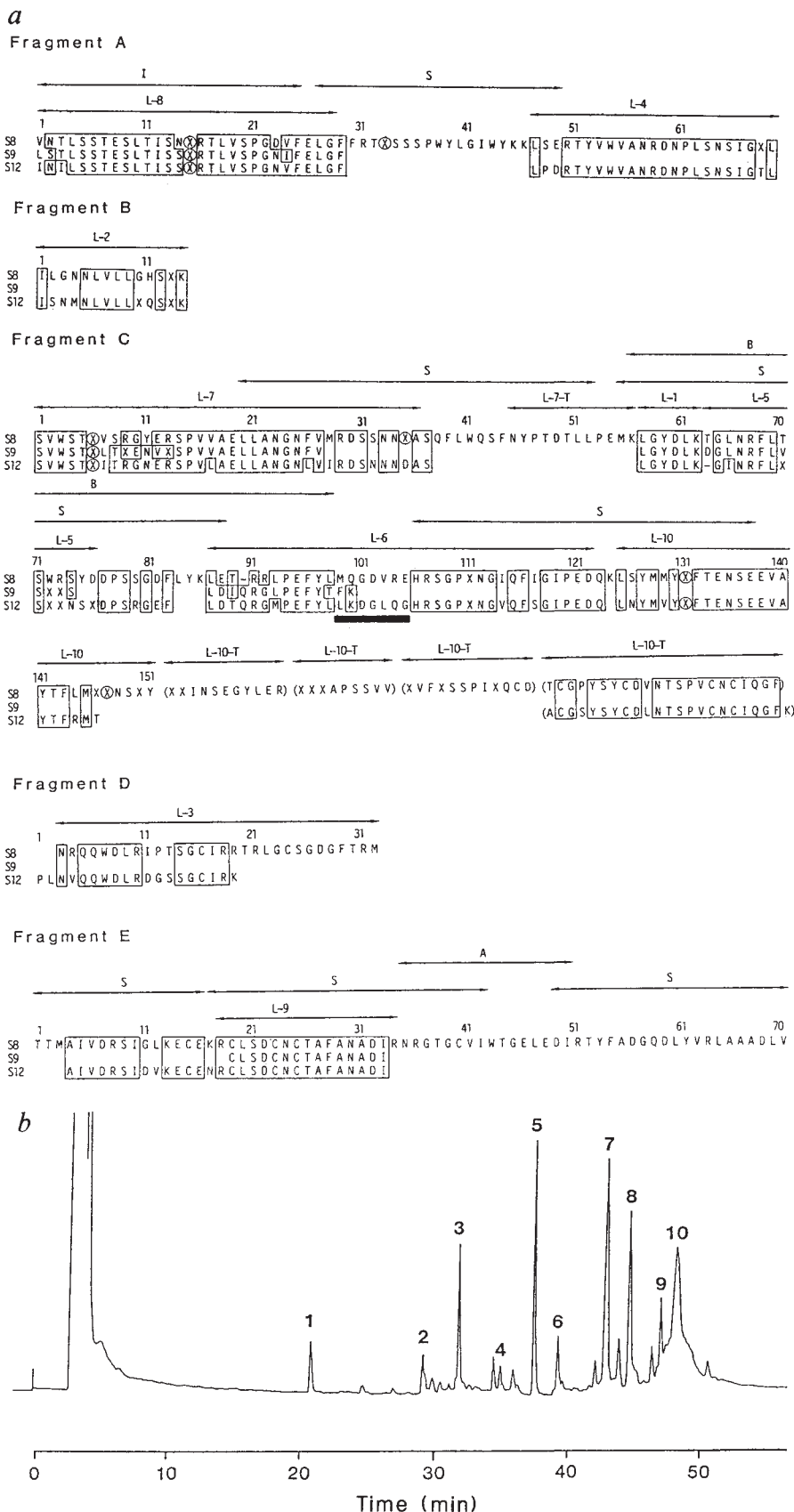
Immunological analyses suggest that the *S*-glycoproteins of both the two *Brassica* species have a common structure<sup>5</sup>. We have found that the amino-terminal sequence of the *S*<sub>9</sub>-glycoprotein of *B. oleracea*<sup>14</sup> is INTLSXTESLX1, which is very similar to the corresponding amino terminus of *B. campestris*. These data strongly suggest that the *S*-glycoproteins of these *Brassica* species are mutually homologous and that the amino-terminal residue of *S*<sub>6</sub>-glycoprotein is isoleucine at the amino acid 14 of the protein sequence deduced from the cDNA. The peptide before the amino terminus of *S*<sub>6</sub>-glycoprotein (dashed line in Fig. 2), with a sequence characteristic of a signal peptide<sup>15</sup> should be a carboxy-terminal part of the signal peptide, because the initiation codon methionine was not present in the cDNA sequenced. Therefore, insertion of one base pair each at positions 333–334, 766–767 and 812–813 is the most plausible revision to resolve the contradiction between our peptide sequences and the cDNA of Nasrallah *et al.* It results in a longer open reading frame encoding 405 amino acids and ending at the TAG codon at base-pair position 1,252. Then all the peptide fragments of *S*<sub>8</sub>-glycoprotein can be aligned homologously with the putative amino-acid sequence of *S*<sub>6</sub>-glycoprotein. The amino-acid sequences of these two *S*-glycoproteins show >80% similarity. The part which varies in *S*<sub>8</sub>- and *S*<sub>12</sub>-glycoproteins (underlined in Fig. 1a) was greatly different also in *S*<sub>6</sub>-glycoprotein (underlined with a wide bar in Fig. 2). We shall assume that in reading the base pairs at three positions in the published cDNA sequence of the *S*<sub>6</sub>-glycoprotein, a base pair was inadvertently omitted from the sequence, as has occasionally occurred.

We propose the primary structure of *S*<sub>8</sub>-glycoprotein drawn schematically in Fig. 3a. The putative amino-acid sequence of *S*<sub>6</sub>-glycoprotein had nine potential glycosylation sites and they may be almost the same as those in *S*<sub>8</sub>-glycoprotein shown in Fig. 2. Six of the nine sites were thought to be glycosylated in the *S*<sub>8</sub>-glycoprotein because no PTH-amino acids were detected



**Fig. 1** Determination of amino-acid sequence. *a*, Amino-acid sequences of  $S_8$ -,  $S_9$ -,  $S_{12}$ -glycoproteins as fragments from *B. campestris*. Peptides were obtained by cyanogen bromide fragmentation and digestion of  $S_8$ -glycoprotein with proteolytic enzymes. The sequences of the intact glycoprotein and the peptide fragments determined by sequence analysis with gas-phase micro sequencer (Applied Biosystems 470A) are shown as solid lines above the sequence. Letters refer to (I) intact fragments and those digested with (L) lysyl endopeptidase, (T) trypsin, (S) *Staphylococcus aureus* V8 protease, (A) endoproteinase Arg C and (B) cyanogen bromide. The numbers following L are fragment numbers obtained from preparative HPLC (see *b*). The L-7-T and L-10-T sequences are the trypsin digestion fragments of L-7 and L-10 fragments, respectively. All the peptide sequences of  $S_9$ - and  $S_{12}$ -glycoproteins were obtained from their lysyl endopeptidase peptides and arranged under the homologous sequences of  $S_8$ -glycoprotein. In fragment E two V8 protease peptides 1-16 and 17-42 in the  $S_8$ -glycoprotein are linked because of a homologous lysyl endopeptidase fragment, 14-33, of the  $S_{12}$ -glycoprotein. Each amino acid is designated by one letter (A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr). Unidentified residues are indicated by X, including the glycosylated asparaginyl residue; circled X is a potential glycosylation site. Gaps indicated by hyphens are introduced to optimize alignment of similar regions. Residues the same in more than two  $S$ -glycoproteins are boxed. *b*, HPLC chromatogram of lysyl endopeptidase fragments of  $S_8$ -glycoprotein after modification of disulphide bonds with iodoacetamide.

**Methods.** The S-glycoproteins were purified from stigmas of  $S_8S_8$ ,  $S_9S_9$  and  $S_{12}S_{12}$  homozygotes of *B. campestris* by a combination of Sephadex G-100 gel filtration, concanavalin A/Sepharose affinity chromatography, ion-exchange HPLC and reverse-phase HPLC<sup>11</sup>. In each step of the purification, the S-glycoproteins were followed by monitoring the appropriate band on polyacrylamide isoelectric focusing gels (isoelectric points for  $S_8$ ,  $S_9$  and  $S_{12}$  were 8.8, 6.9 and 5.7, respectively). For proteolytic digestion, the cysteinyl residues were first reduced and alkylated. S-glycoprotein (40–80  $\mu$ g) was dissolved in 30  $\mu$ l of deoxygenated 0.5 M Tris HCl/6M urea (pH 8.5). Dithiothreitol was added to a final concentration of 4–6 mM and incubated at 45°C for 1 h. Then a solution (10  $\mu$ l) of either 40 mM iodoacetamide or vinylpyridine was added and the incubation was continued in the dark at room temperature for 20 min. The alkylation was terminated by adding a solution (10  $\mu$ l) of 40 mM cysteine hydrochloride. The reaction mixture was diluted with water to 400  $\mu$ l and subjected to the following proteolytic digestion without further purification. The enzymes, their concentrations and other conditions used were as follows. Lysyl endopeptidase (Wakojunyaku): 1:20 molar ratio of enzyme to substrate, 37°C, 24 h; L-L-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma): 1:20 (w/w), 37°C, 3 h; *Staph. aureus* V8 protease (Miles): 1:15 (w/w), 37°C, 20 h; endoproteinase Arg C (Sigma): 1:10 (w/w), 37°C, 3 h. In each case the enzymatic digestion was stopped by adding 1–2  $\mu$ l of formic acid. A cysteine-rich fragment obtained by treating with lysyl endopeptidase was further cleaved by trypsin yielding several small fragments. For cyanogen bromide digestion,  $S_8$ -glycoprotein (67  $\mu$ g) was first dissolved in 50  $\mu$ l of 70% formic acid. A 100-fold excess of cyanogen bromide was added and the incubation was continued overnight in the dark at room temperature. The reaction mixture was then diluted ten times with water and lyophilized. All digestion fragments were fractionated on a Hi-Pore RP-304 column (4.6  $\times$  250 mm, Bio-Rad) with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid (60 min). The peptide fragments were detected by UV absorbance at 220 nm. Amino-acid sequencing was a sequencing cycle was identified by an on-line PTH-an-



|            |     |                                                                                                    |            |                                                                                 |                                                                                                    |
|------------|-----|----------------------------------------------------------------------------------------------------|------------|---------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|
| cDNA<br>S6 | 1   | GGG, TCC, GTC, GTC, TTG, ATT, CTA, TTT, TGT, CCT, GCC, TTT, TCG, ATC, AAC, ACT, TTG, TCG, TCT, ACA | cDNA<br>S6 | 660                                                                             | CTG, AGT, TAC, ATG, GTG, TAC, AAC, TTC, ACA, GAG, AAT, AGT, GAA, GAG, GTC, GCT, TAT, ACA, TTC, CGA |
| -13        |     | Gly Ser Val Val Leu Ile Leu Phe Cys Pro Ala Phe Ser Ile Asn Thr Leu Ser Ser Thr                    | 208        | Leu Ser Tyr Met Val Tyr Asn Phe Thr Glu Asn Ser Glu Glu Val Ala Tyr Thr Phe Arg |                                                                                                    |
| S8         | 1   | Val Asn Thr Leu Ser Ser Thr                                                                        | S8         | 208                                                                             | Leu Ser Tyr Met Met Tyr X Phe Thr Glu Asn Ser Glu Glu Val Ala Tyr Thr Phe Leu                      |
|            |     | A                                                                                                  |            |                                                                                 |                                                                                                    |
| cDNA<br>S6 | 61  | GAA, TCT, CTT, AGA, ATC, TCA, AGC, AAC, AGA, ACA, CTT, GTA, TCT, CCA, GGT, AAT, AAC, TTC, GAA, CTC | cDNA<br>S6 | 720                                                                             | ATG, ACC, AAC, AAC, AGC, ATC, TAC, TCG, AGA, TTG, ACA, CTA, AGT, TCC, GAA, GGC, TAT, TTT, CAG, CGA |
| 8          |     | Glu Ser Leu Arg Ile Ser Ser Asn Arg Thr Leu Val Ser Pro Gly Asn Asn Phe Glu Leu                    | 228        | Met Thr Asn Asn Ser Ile Tyr Ser Arg Leu Thr Leu Ser Ser Glu Gly Tyr Phe Gln Arg |                                                                                                    |
| S8         | 8   | Glu Ser Leu Thr Ile Ser Asn X Arg Thr Leu Val Ser Pro Gly Asp Val Phe Glu Leu                      | S8         | 228                                                                             | Met X X Asn Ser X Tyr X X Ile Asn Ser Glu Gly Tyr Leu Glu Arg                                      |
|            |     |                                                                                                    |            |                                                                                 |                                                                                                    |
| cDNA<br>S6 | 121 | GGC, TTC, TTC, CGA, ACC, AAC, TCA, AGT, TCT, CGT, TGG, TAT, CTC, GGG, ATA, TGG, TAC, AAG, AAA, TTG | cDNA<br>S6 | 779                                                                             | CTT, ACG, TGG, AAT, CCG, TCA, ATA, GGG, ATA, TGG, ACA, GGC, TTC, TGG, TCT, TCT, CCA, GTG, GAC, CCC |
| 28         |     | Gly Phe Phe Arg Thr Asn Ser Ser Ser Arg Trp Tyr Leu Gly Ile Trp Tyr Lys Lys Leu                    | 248        | Leu Thr Trp Asn Pro Ser Ile Gly Ile Trp Thr Ala Phe Thr Ser Ser Pro Val Asp Pro |                                                                                                    |
| S8         | 28  | Gly Phe Phe Arg Thr X Ser Ser Ser Pro Trp Tyr Leu Gly Tyr Trp Tyr Lys Lys Leu                      | S8         | 248                                                                             | X X X Ala Pro Ser Ser Val Val X Val Phe X Ser Ser Pro Ile - X                                      |
|            |     |                                                                                                    |            |                                                                                 |                                                                                                    |
| cDNA<br>S6 | 181 | CTC, GAC, AGA, ACC, TAT, GTA, TGG, GTT, GCC, AAC, AGA, GAT, AAC, CCA, CTC, TCC, AAT, GCC, ATT, GGA | cDNA<br>S6 | 838                                                                             | CAG, TGC, GAT, ACA, TAC, ATA, ATG, TGC, GGG, CCT, TAC, GCT, TAC, TGT, GGC, GTG, AAC, ACA, TCA, CCT |
| 48         |     | Leu Asp Arg Thr Tyr Val Trp Val Ala Asn Arg Asp Asn Pro Leu Ser Asn Ala Ile Gly                    | 268        | Gln Cys Asp Thr Tyr Ile Met Cys Gly Pro Tyr Ala Tyr Cys Gly Val Asn Thr Ser Pro |                                                                                                    |
| S8         | 48  | Ser Glu Arg Thr Tyr Val Trp Asn Ala Asn Arg Asp Asn Pro Leu Ser Asn Ser Ile Gly                    | S8         | 268                                                                             | Gln Cys Asp Thr Cys Gly Pro Tyr Ser Tyr Cys Asp Val Asn Thr Ser Pro                                |
|            |     |                                                                                                    |            |                                                                                 |                                                                                                    |
| cDNA<br>S6 | 241 | ACC, CTC, AAA, ATC, TCA, GGC, AAT, AAT, CTT, GTC, CTC, CTT, GGT, CAC, ACC, AAT, AAA, TCT, GTT, TGG | cDNA<br>S6 | 898                                                                             | GTT, TGT, AAC, TGT, ATC, CAA, GGG, TTC, AAT, CCC, CGG, AAT, ATA, CAG, CAG, TGG, GAT, CAG, AGA, GTC |
| 68         |     | Thr Leu Lys Ile Ser Gly Asn Asn Leu Val Leu Leu Gly His Thr Asn Lys Ser Val Trp                    | 288        | Val Cys Asn Cys Ile Gln Gly Phe Asn Pro Arg Asn Ile Gln Gln Trp Asp Gln Arg Val |                                                                                                    |
| S8         | 68  | X Leu Ile Leu Gly Asn Asn Leu Val Leu Leu Gly His Ser X Lys Ser Val Trp                            | S8         | 288                                                                             | Val Cys Asn Cys Ile Gln Gly Phe Asn Arg Gln Gln Trp Asp Leu Arg Ile                                |
|            |     |                                                                                                    |            |                                                                                 |                                                                                                    |
| cDNA<br>S6 | 301 | TCG, ACG, AAT, CTT, ACT, AGA, GGA, AAT, GAG, AGA, CTT, CCG, GTG, GTG, GCA, GAC, GTT, CTC, TCT, AAT | cDNA<br>S6 | 958                                                                             | TGG, GCA, GGT, GGG, TGT, ATA, AGG, AGG, ACG, CGG, CTT, AGC, TGC, AGT, GGA, GAT, GGT, TTT, ACA, AGG |
| 88         |     | Ser Thr Asn Leu Thr Arg Gly Asn Glu Arg Leu Pro Val Val Ala Asp Val Leu Ser Asn                    | 308        | Trp Ala Gly Gly Cys Ile Arg Arg Thr Arg Leu Ser Cys Ser Gly Asp Gly Phe Thr Arg |                                                                                                    |
| S8         | 88  | Ser Thr X Val Ser Arg Gly Tyr Glu Arg Ser Pro Val Val Ala Glu Leu Leu Ala Asn                      | S8         | 308                                                                             | Pro Thr Ser Gly Cys Ile Arg Arg Thr Arg Leu Gly Cys Ser Gly Asp Gly Phe Thr Arg                    |
|            |     |                                                                                                    |            |                                                                                 |                                                                                                    |
| cDNA<br>S6 | 360 | GGA, AAC, TTC, GTG, ATG, CGA, GAC, TCC, AGT, AAC, AAC, GAC, GCA, AGT, GAA, TAC, TTG, TGG, CAA, AGT | cDNA<br>S6 | 1018                                                                            | ATG, AAG, AAC, ATG, AAG, CTG, CCA, GAA, ACT, ACG, ATG, GCG, ATT, GTC, GAC, CGC, AGT, ATT, GGT, GTG |
| 108        |     | Gly Asn Phe Val Met Arg Asp Ser Ser Asn Asn Asp Ala Ser Glu Tyr Leu Trp Gln Ser                    | 328        | Met Lys Asn Met Lys Leu Pro Glu Thr Thr Met Ala Ile Val Asp Arg Ser Ile Gly Val |                                                                                                    |
| S8         | 108 | Gly Asn Phe Val Met Arg Asp Ser Ser Asn Asn X Ala Ser Gln Phe Leu Trp Gln Ser                      | S8         | 328                                                                             | Met Thr Thr Met Ala Ile Val Asp Arg Ser Ile Gly Leu                                                |
|            |     |                                                                                                    |            |                                                                                 |                                                                                                    |
| cDNA<br>S6 | 420 | TTC, GAT, TAC, CCT, ACG, GAT, ACT, TTG, CTT, CCA, GAG, ATG, AAA, CTG, GGT, TAC, GAC, CTC, AAA, ACA | cDNA<br>S6 | 1078                                                                            | AAA, GAA, TGT, GAG, AAG, AGG, TGC, CTT, AGC, GAT, TGT, AAT, TGT, ACT, GCT, TTT, GCA, AAT, GCG, GAT |
| 128        |     | Phe Asp Tyr Pro Thr Asp Thr Leu Leu Pro Glu Met Lys Leu Gly Tyr Asp Leu Lys Thr                    | 348        | Lys Glu Cys Glu Lys Arg Cys Leu Ser Asp Cys Asn Cys Thr Ala Phe Ala Asn Ala Asp |                                                                                                    |
| S8         | 128 | Phe Asn Tyr Pro Thr Asp Thr Leu Leu Pro Glu Met Lys Leu Gly Tyr Asp Leu Lys Thr                    | S8         | 348                                                                             | Lys Glu Cys Glu Lys Arg Cys Leu Ser Asp Cys Asn Cys Thr Ala Phe Ala Asn Ala Asp                    |
|            |     |                                                                                                    |            |                                                                                 |                                                                                                    |
| cDNA<br>S6 | 480 | GGG, TTG, AAC, AGG, TTC, CTT, ACA, TCA, TGG, AGA, AGT, TCA, GAT, GAT, CCA, TCA, AGC, GGG, GAT, TTC | cDNA<br>S6 | 1138                                                                            | ATC, CGG, AAT, GGT, GGG, ACG, GGT, TGT, GTG, ATT, TGG, ACC, GGA, CGG, CTT, GAC, GAT, ATG, CGG, AAT |
| 148        |     | Gly Leu Asn Arg Phe Leu Thr Ser Trp Arg Ser Trp Asp Asp Pro Ser Ser Gly Asp Phe                    | 368        | Ile Arg Asn Gly Thr Gly Cys Val Ile Trp Thr Gly Arg Leu Asp Asp Met Arg Asn     |                                                                                                    |
| S8         | 148 | Gly Leu Asn Arg Phe Leu Thr Ser Trp Arg Ser Trp Asp Asp Pro Ser Ser Gly Asp Phe                    | S8         | 368                                                                             | Ile Arg Asn Arg Gly Thr Gly Cys Val Ile Trp Thr Gly Glu Leu Glu Asp Ile Arg Thr                    |
|            |     |                                                                                                    |            |                                                                                 |                                                                                                    |
| cDNA<br>S6 | 540 | TCG, TAC, AAG, CTC, GAA, ACC, CGA, AGC, CTT, CCT, GAG, TTT, TAT, CTA, TGG, CAT, GGG, ATC, TTT, CCA | cDNA<br>S6 | 1198                                                                            | TAC, GTT, GCT, CAC, GGT, CAA, GAT, CTT, TAT, GTC, AGA, TTG, GCT, GTT, GCT, GAC, CTT, GTT, TAG, CTC |
| 168        |     | Ser Tyr Lys Leu Glu Thr Arg Ser Leu Pro Glu Phe Tyr Leu Trp His Gly Ile Phe Pro                    | 388        | Tyr Val Ala His Gly Gln Asp Leu Tyr Val Arg Leu Ala Val Ala Asp Leu Val ***     |                                                                                                    |
| S8         | 168 | Leu Tyr Lys Leu Glu Thr Arg Arg Leu Pro Glu Phe Tyr Leu Met Gln Gly Asp Val Arg                    | S8         | 388                                                                             | Tyr Phe Ala Asp Gly Gln Asp Leu Tyr Val Arg Leu Ala Ala Ala Asp Leu Val                            |
|            |     |                                                                                                    |            |                                                                                 |                                                                                                    |
| cDNA<br>S6 | 600 | ATG, CAT, CCG, AGT, GGT, CCA, TGG, AAT, GGA, GTC, CGA, TTT, AGT, GGC, ATA, CCA, GAG, GAC, CAA, AAG | cDNA       | 1258                                                                            | TTTCTCTAAATAAGCAGCGATGCC 1293                                                                      |
| 188        |     | Met His Arg Ser Gly Pro Trp Asn Gly Val Arg Phe Ser Gly Ile Pro Glu Asp Gln Lys                    |            |                                                                                 |                                                                                                    |
| S8         | 188 | Glu His Arg Ser Gly Pro Trp Asn Gly Ile Gln Phe Ile Gly Ile Pro Glu Asp Gln Lys                    |            |                                                                                 |                                                                                                    |

**Fig. 2** Revised cDNA sequence and the deduced amino-acid sequence of  $S_6$ -glycoprotein, and the alignment of the predicted sequences with  $S_7$ -glycoprotein amino-acid sequence. The cDNA and amino acids of two proteins are numbered on the left. Top sequence, cDNA: the revised cDNA sequence of the  $S_6$ -glycoprotein. Position numbers are unchanged from the published data<sup>9</sup>, and the inserted bases (tentatively C) are marked with vertical arrows. The codon with the wavy line over it indicates the TGA codon proposed as the termination codon in the published data<sup>9</sup>. Arrows and asterisks below nucleotide sequence, potential polyadenylation signal site<sup>19</sup>. S6: the amino-acid sequence of the  $S_6$ -glycoprotein deduced from the above cDNA. Dashed line, proposed part of the signal peptide. The termination codon of the revised cDNA sequence is marked with asterisks in this line. Asn residues marked with open triangles are the potential glycosylation sites deduced from the cDNA sequence of  $S_6$ -glycoprotein. S8: determined amino-acid sequence of  $S_8$ -glycoprotein. Bold-type capitals under the sequences show the peptide fragments in Fig. 1a. X, unidentified amino acid; X marked with closed triangle, potential glycosylation sites deduced from amino-acid sequencing. Identities in the sequences between  $S_6$ - and  $S_8$ -glycoproteins are marked with two dots between the two lines. Wide bar, variable region which differs between the  $S$ -glycoproteins of *B. campestris*, and this part was also different in the  $S_6$ -glycoprotein. Twelve cysteine residues are marked with large stars.

in these cycles. Replacements of glycosylated asparagine were observed at amino acids 119 and 251. The estimated  $M_r$  of  $S_8$ -glycoprotein (53K) corresponds to the sum of 405 amino acids (46K) and 7 oligosaccharide chains.

Oligosaccharides A and B were the main components of  $N$ -glycosidic carbohydrate chains in  $S$ -glycoprotein (Fig. 3b). Oligosaccharide A ( $M_r$  1,391) was predominant and may be the mature form.

The twelve cysteine residues located at the carboxy-terminal end (Fig. 3a) should form six disulphide bonds, so this region would form a rigid cluster. In this region, two potential glycosylation sites at amino acids 284 and 359 in  $S_6$ -glycoprotein may not be glycosylated as in  $S_8$ -glycoprotein. The hydrophilicity index calculated by the method of Hopp and Woods<sup>16</sup> for  $S_6$ - and  $S_8$ -glycoproteins showed a similar pattern and no highly hydrophobic zone indicating a transmembrane sequence was observed.

Amino-acid sequence similarity has been reported among  $S$ -glycoproteins of the gametophytic self-incompatibility sys-

tems of *Nicotiana glauca* ( $S_2$ -glycoprotein) and of *Lycopersicon peruvianum*<sup>10</sup> ( $S_1$ - and  $S_3$ -glycoproteins). The homology reported here is among  $S$ -glycoproteins of *Brassica* species, which have sporophytic incompatibility systems. The gametophytic system is generally considered to be the ancestral form of self-incompatibility in flowering plants, with the sporophytic system being derived from it<sup>1</sup>. So homology might be anticipated between the  $S$ -glycoproteins of *N. glauca* and *Brassica* species. But between two  $S$ -glycoproteins of the sporophytic and gametophytic self-incompatibility systems, no amino-acid sequence homology is observed.

A search for peptides homologous to  $S$ -glycoproteins of *Brassica* species in a protein database by Gotoh's method<sup>17</sup> indicated that the sequence of the cysteine-rich part has some homology with a family of epidermal growth factor<sup>18</sup>. Whether this is just accidental or has some meaning is not clear.

We thank Dr O. Gotoh of Saitama Cancer Center Research Institute for the use of his system for searching a protein database and to Dr H. Ozawa of the Computer Center of the University



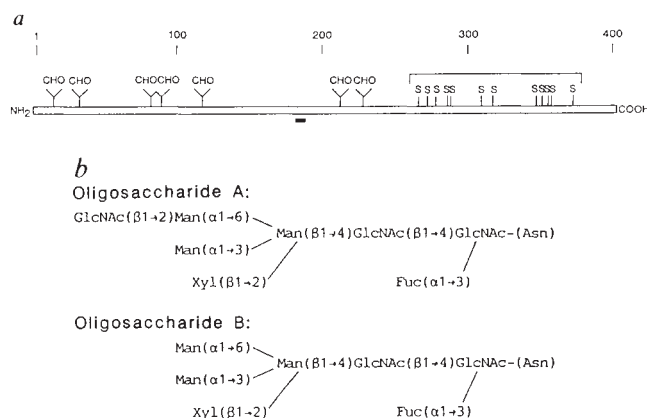
## Erratum

## Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement

Paul J. Godowski, Sandro Rusconi, Roger Miesfeld &amp; Keith R. Yamamoto

*Nature* 325, 365-368 (1987).

IN this letter Figs 3 and 4 were printed incorrectly, without the arrows referred to in the legends. The figures appear correctly below.

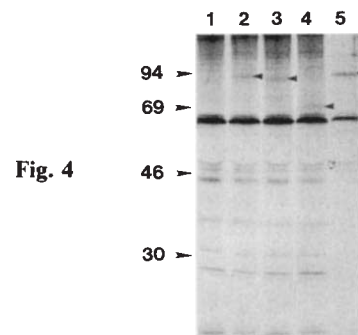


**Fig. 3** Schematic drawing of *S*-glycoproteins. **a**, Schematic drawing of the *S*<sub>8</sub>-glycoprotein of *B. campestris*. CHO, carbohydrate chain; S, cysteinyl residue. Heavy bar, zone variable among *Brassica* species. **b**, Structures of oligosaccharides A and B in *S*-glycoproteins of *B. campestris*, oligosaccharide A being predominant. The identity of the major oligosaccharide chains of total stigma proteins with those of *S*-glycoprotein was confirmed by the sequential digestion experiment of the isolated pyridylamino-derivatized saccharide chains from *S*-glycoproteins with various exoglycosidases in comparison with those from the total stigma glycoproteins and from bromelain. The details of the structural elucidation of the saccharide chains of *S*-glycoproteins of *B. campestris* will be published elsewhere (in preparation).

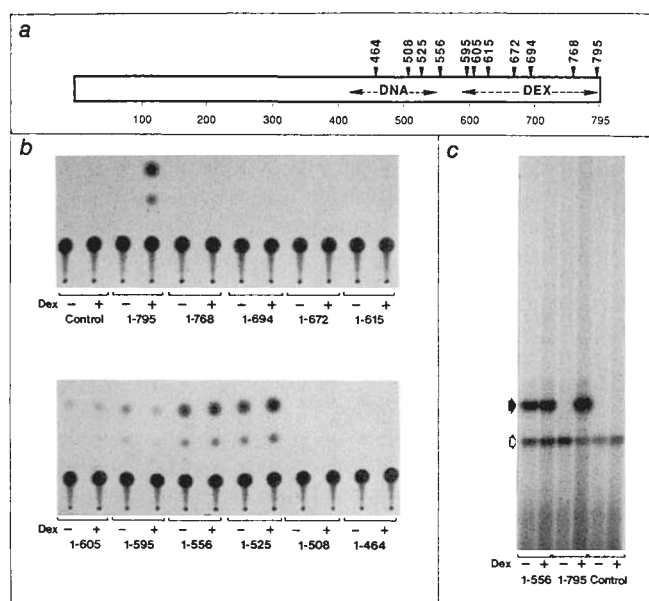
of Tokyo for permitting us to use his database file. We also thank Professor T. Blundell of University of London for his critical reading of the manuscript. This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (Nos 60125004 and 61117002).

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**Fig. 4**



**Fig. 3**

## Hyperacuity and the visual cortex

RECENTLY Swindale and Cynader<sup>1</sup> demonstrated experimentally that single neurons in cat visual cortex show a form of hyperacuity. They drew several comparisons with our own measurements of hyperacuity in the monkey visual cortex and with our theoretical analysis<sup>2</sup>. They (and Martin<sup>3</sup>) support the presumption that the limiting factors in hyperacuity, in particular Vernier acuity, are purely cor-

tical, in contrast to the limiting factors in resolution acuity, which are thought to be essentially retinal.

Their results showed that a moving Vernier target caused maximum stimulation of a cortical cell when the bars of the target were exactly collinear. Progressively weaker responses were obtained when the bars were offset in a direction orthogonal to their long axes. Significant decrements in response occurred with offsets smaller than the overall size of the cortical cell's receptive field. They argued that this

demonstrated a hyperacuity for relative position of the two bars and that this phenomenon was uniquely cortical.

Suppose the moving Vernier target were to stimulate a very simple model receptive field, consisting of a single excitatory region and simple temporal integrator. Results very similar to those of Swindale and Cynader would be obtained; this would be attributable to the fact that only the collinear version of the Vernier target provides optimal, synchronous stimulation of the receptive field in space and

## MATTERS ARISING

time. A tuning curve for Vernier offset could also be obtained by flashing the targets on the receptive field. It seems premature to claim that such results in cortical cells demonstrate a specific sensitivity for relative position when a simpler explanation based on sensitivity for absolute position is possible.

In the case of cat retinal ganglion cells, experiments have revealed thresholds as low as 1 arc min for simple positional sensitivity and models based on linear summation account for these results<sup>4-6</sup>. For monkey cortical cells our own measurements and theoretical predictions<sup>2</sup> showed positional thresholds as low as 10-20 arc s. With the addition of a temporal component<sup>3</sup>, these models also predict the kind of results obtained by Swindale and Cynader in cortical cells.

Indeed, all the current results for cortical cells in hyperacuity tasks are readily predicted from similar linear models of receptive field organization. There is good evidence that linear models can be applied to the spatial summation properties of cortical simple cells and to the linearly-summing subunits of complex cells<sup>7-9</sup>. If a specific nonlinear mechanism for extracting relative position were to be demonstrated in cortical cells this would be highly significant, but present evidence indicates that cortical cells have thresholds in the hyperacuity range by virtue of essentially the same type of mechanism that is present in retinal ganglion cells. This similarity is reinforced by the recognition that the firing of a striate cortical cell is ambiguous in much the same way that the firing of a retinal ganglion cell is ambiguous. Changes of contrast, position, spatial frequency, orientation and direction of drift are all effective in altering the firing rate of cortical neurons.

For these reasons, it is also mistaken to conclude that the existence of cortical neurons with positional sensitivity in the hyperacuity range is an argument against the existence of a fine-grain spatial reconstruction<sup>10</sup>. Strictly speaking, current neurophysiological data do not address this question directly. Psychophysics has pointed to the importance of interpolation over a spatially-sampled luminance profile and to the necessity of constructing an exact signal for spatial location, but it is an open question whether this is accomplished by the construction of a fine-grain spatial map or by other means.

The most interesting feature of comparing the hyperacuity performance of retinal and cortical neurons is that the cortical transformation of information preserves very well the accuracy of the positional signals supplied by the retina, even though cortical cells acquire new forms of selectivity, such as orientation and binocularity. The existence of single neurons at several levels in the visual system with thresholds close to those of a psychophy-

sical observer strikingly demonstrates the precision of organization of the early visual pathways and the high quality of the 'components' used for visual computation.

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**SWINDALE AND CYNADER REPLY**—It is obvious that the information required to make hyperacuity judgements must be present in the signals from the retina. As such judgements can be made for spots of light that stimulate only small numbers of retinal ganglion cells<sup>1</sup>, it is not surprising that absolute positional sensitivity in retinal neurons can be as good as the sensitivity to Vernier offset demonstrated behaviourally. The fact that calculated retinal positional sensitivities<sup>2,3</sup> may be even higher than those needed to account for behavioural hyperacuity implies, as Martin suggested<sup>4</sup>, that the limiting factors in hyperacuity are cortical and not retinal in origin. Thus it makes sense to study hyperacuity in the cortex and not, as Parker and Hawken would have us do, in the retina.

Although retinal signals contain the information about absolute position required for hyperacuity judgements, such judgements involve comparisons of the positions of features that are further apart than the sizes of ganglion cell receptive fields<sup>5</sup>. Furthermore, changes in the retinal position of the stimulus (caused for example by eye movements) during or between successive presentations, do not degrade acuity<sup>5</sup>. For both these reasons it is unlikely that a signal from the type of retinal detector hypothesised by Parker and Hawken will, on its own, be much use as a basis for a hyperacuity discrimination.

The claim that our results are "readily predicted" from linear models of receptive field organization is not supported. We know of no theoretical predictions of the acuity of retinal or cortical neurons for moving Vernier stimuli. Our preliminary modelling indicates that linear summation within a simple cell's receptive field would produce tuning curves flatter than those observed, and the same is almost certain

to be true for retinal cells. There is however experimental evidence for non-linearities of integration along the receptive field axis of simple cells<sup>6-8</sup> which could increase the Vernier sensitivity of an otherwise linear cell.

It is true that the firing rate of a cortical cell is an ambiguous signal but it is certainly less ambiguous than the firing of a retinal cell, by virtue of the cortical cell's feature selectivity. In any case, these ambiguities may not pose as serious a problem for relating cortical neuronal responses to psychophysics as one might suppose. In psychophysical experiments, potentially confounding variables such as the brightness, contrast, length and velocity of the bars in a Vernier target are normally kept constant, and it is possible that random variations in these parameters would indeed degrade performance. If such variations were present it might still be possible to disambiguate a response by combining signals from cells (for example, by subtracting the signals from cells with the same contrast sensitivity, but different Vernier sensitivities).

There are other reasons besides our experimental results (or our interpretation of them) for supposing that a fine-grain representation of receptive field position is not a necessary prerequisite for explaining hyperacuity. One is that the receptive field sizes of the cells involved in such a representation will be no smaller than those of the more coarsely spaced fields of the cells that form the input. Their positional sensitivities will be no greater than that of their inputs, and it is not clear how this extra stage would help the subsequent extraction of information about relative position. The other evidence is the lack of a neuronal substrate for a fine grain representation in the cat: there is no evidence for a layer of closely packed cells with small non-oriented receptive fields as there is in the monkey. Nevertheless, cats have hyperacuity.

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## Matters Arising

Future contributions to *Matters Arising* should instead be sent to Scientific Correspondence and need not be sent to the authors of the original article in advance. Priority will be given to letters of less than 500 words and five references.



# Tunnelling reveals forbidden transitions

K.W. Hipps

*Inelastic electron tunnelling spectroscopy provides unique information about vibrational and electronic transitions that are forbidden to photon-induced processes.*

INELASTIC electron tunnelling spectroscopy (IETS) was first developed by Lambe and Jaklevic<sup>1</sup> in 1966. Since that time the technique has evolved and applications now range from the study of biochemicals to transition metal complexes. Several reviews and texts are available<sup>2-4</sup>. IETS is a non-optical method for measuring vibrational and electronic spectra. It is a very sensitive technique, providing the spectrum of a sample containing less than  $10^{13}$  molecules. It has been heavily used for the study of chemisorbed species, as a molecular spectroscopy complementary to infrared (IR) and Raman, and for the observation of transitions which are 'silent' (forbidden) in photon spectroscopy<sup>1-9</sup>. The most unusual feature of the technique is that the sample of interest must be incorporated into a metal-insulator-metal thin film device called a tunnel diode. Thus, sample preparation is more of an art than with IR and Raman spectroscopy. The following discussion describes the use of IETS for the observation of optically forbidden transitions.

## More on the method

If two sheets of metal are separated by a thick ( $>50$  nm) insulator, a capacitor results, and the application of a steady potential difference between the plates produces no steady current flow. As the thickness of the insulator is reduced below about 5 nm, however, current does flow and the device has a measurable conductance. This current is due to a quantum mechanical phenomenon known as tunnelling. The electron transmission through the insulator is such that the electron's total energy is less than its potential energy in the insulator (barrier) region. Thus, the electrons seem to be 'tunnelling' through the potential barrier. If the electron moves from the first metal to the second without loss of total energy, the process is termed elastic tunnelling. This is the primary conduction mechanism for tunnel diodes. An inelastic process may also occur. In this case, a portion of the electron's translation energy is lost to the barrier region in the form of vibrational or electronic excitation. It is this inelastic tunnelling process which provides the physical basis for IETS.

Taking  $I$  as the current flowing in the junction due to applied (bias) voltage  $V$ , a plot of  $(d^2I/dV^2)$  versus  $V$  yields a tunnelling spectrum<sup>1,2</sup>. Peaks occur at energies (1

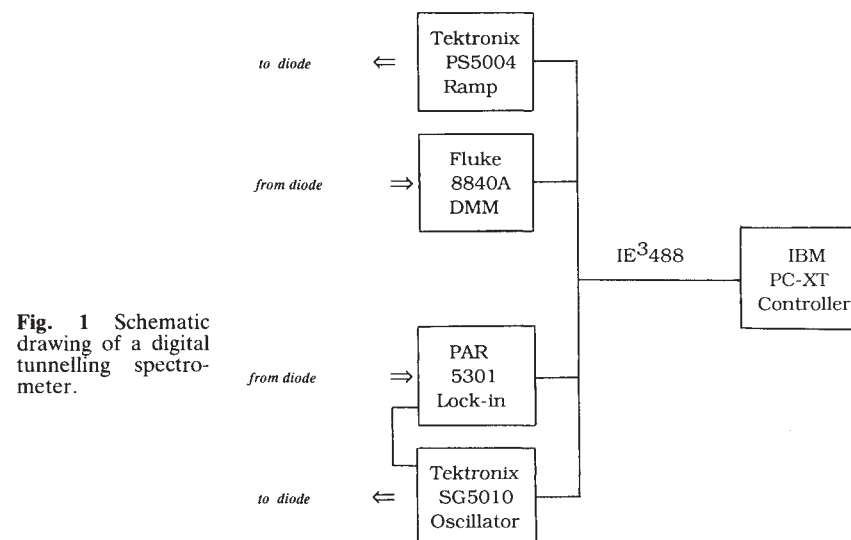


Fig. 1 Schematic drawing of a digital tunnelling spectrometer.

volt =  $8.066 \text{ cm}^{-1}$ ) corresponding to electronic or vibrational transitions. The band widths depend strongly on the temperature, and are typically about  $15 \text{ cm}^{-1}$ , at 4.2 K. Most tunnelling spectra, therefore, are taken at, or below, liquid helium temperature. The intensities of the observed spectra depend on electron-molecule interactions and are not well understood. It is known empirically that Raman active, electric and magnetic dipole active, and optically forbidden transitions may be observed. Transitions having  $\Delta S = \pm 1$  and 0 are also observed.

At present, no tunnelling spectrometer is commercially available. There are however, a number of different designs described in the literature<sup>1-5</sup>. The most modern and easily assembled is an all digital spectrometer utilizing an IEEE-488 interface and an IBM-PC-XT controller<sup>5</sup>. A schematic of this spectrometer is shown in Fig 1. In addition to the spectrometer, a high vacuum deposition system is also required to prepare the tunnel diode. Most are prepared in the following manner. A metal film ( $\approx 100$  nm thick  $\times 1$  mm  $\times 20$  mm) is first deposited on a smooth substrate. An insulating material is then grown or deposited on the metal to form a barrier roughly 1.5 nm thick. The species of interest is placed on the insulator to provide a coverage of about one monolayer. This latter step may be done either from the gas phase or from solution. The second metal electrode is then deposited to complete the tunnel junction. Electrical leads are attached to the metal electrodes, the junction is cooled to 4 K or below, and the spectrum is measured.

## Forbidden transitions

In the case of molecular or ionic species having symmetry  $C_{3v}$  or higher, there are generally one or more vibrational modes which are not directly observable by either IR or Raman spectroscopy. The number of parameters required to specify the quadratic potential function for atomic motion greatly exceeds the number of observed vibrations in these systems. Thus, the identification of these missing frequencies can significantly improve our understanding of intramolecular forces. Tunnelling spectroscopy can be used to directly observe many of these 'silent' modes.

Figure 2 shows the low energy portion of the tunnelling spectrum of the octahedral symmetry ferrocyanide ion<sup>9</sup>. Also shown is the IR transmission spectrum of the potassium salt of the same ion. The IET spectrum is much richer in strong bands and has similar band widths. Of special interest are the bands at  $354$  and  $474 \text{ cm}^{-1}$ . These are due to fundamental motions of the ion which are not observable in either IR or Raman spectroscopy. Another example is provided by the  $D_{3h}$  symmetry tricyanomethanide ion  $[\text{C}(\text{CN})_3]^-$ <sup>8</sup>. In this case there is a single in-plane motion which is silent in both IR and Raman. It appears as a fairly intense band in IETS at  $402 \text{ cm}^{-1}$ .

IETS may also be used to study electronic transitions occurring in the spectral region below about 2.5 eV ( $20,000 \text{ cm}^{-1}$ )<sup>2,6</sup>. As in the case of vibrational transitions, both optically allowed and forbidden electronic transitions may be observed.

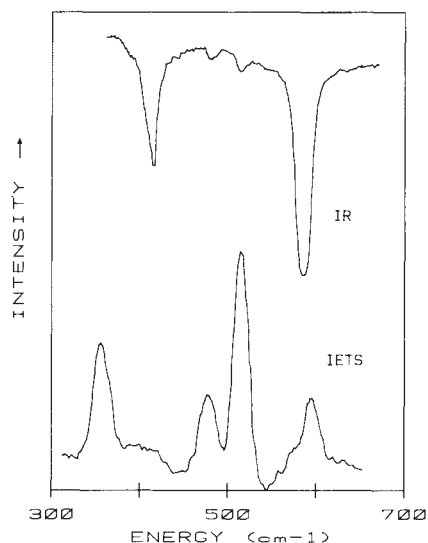


Fig. 2 Tunnelling and infrared spectra of  $K_4Fe(CN)_6$ .

Through IETS it is now possible to begin probing electronic states which have been theoretically predicted but never observed. This should lead to a much better understanding of phenomena such as magnetic susceptibility, thermal properties, photochemistry, and bonding.

### Future directions

There are several problems that limit the application of tunnelling spectroscopy, but these can and will be solved in the future. The most critical need is a wider selection of barrier materials to be used as substrates in IETS. The easily grown alumina and magnesia insulators most commonly used in tunnelling studies are virulent redox agents, but methods for producing ultra-thin and chemically passive insulators are now being developed by several groups of researchers.

A workable theory of IETS intensities is also needed. At the moment it is not even possible to correctly predict the relative intensities of two species differing only by isotopic substitution<sup>7</sup>. Finally, it would be desirable to eliminate the top metal deposition step. In the near future it may be possible to marry the methods of tunnelling spectroscopy to IETS and provide tunnelling spectra as a function of position on the sample surface. □

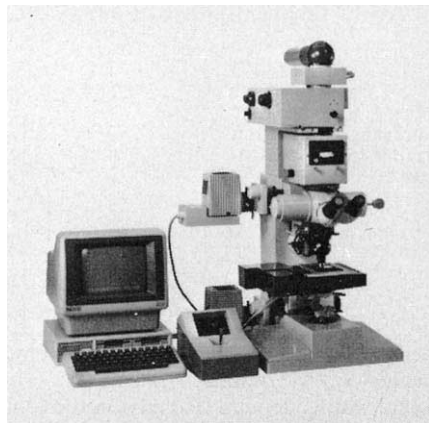
K. W. Hipps is at the Department of Chemistry, Washington State University, Pullman, Washington 99164-4630, USA. For additional information fill in Reader Service No. 100.

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# Spectroscopy specifics

*As one of the largest instrumentation shows, the Pittsburgh Conference, held this year 9-13 March in Atlantic City, New Jersey, has something for every spectroscopist.*

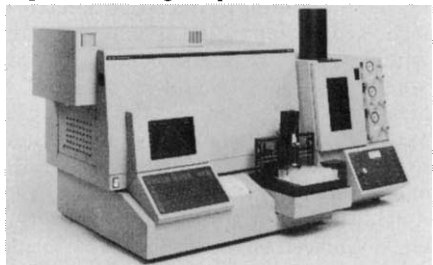
FROM Carl Zeiss, Inc. comes a **compact microscope spectrophotometer** system designed for forensic work in fibre discrimination, lacquer and paint chip analysis, document identification, and counterfeit analysis (Reader Service No. 101). The



*Images as well as analyses samples.*

\$35,000-70,000 (US) Universal Microscope Spectro-Photometer is a modular system for densitometry and spatial analysis of microscopic specimens, and has a spatial resolution in the spectral range from 240 nm (UV) to 2,100 nm (NIR). The system consists of a microscope, photometer, infrared detector, scanning stage and monochromators.

Phillips Analytical has completed its line of spectrometers with its newest offering, the PU 7450 **benchtop inductively coupled plasma spectrometer** (Reader Service No. 102). Phillips says its spectrometer can analyse a wide range of materials from metals and refractories, to geological or biological products. The core of



*Gives complete spectral analysis of a wide range of samples.*

the PU 7450 is an echelle grating monochromator, to provide the resolution required to separate the complex, line-rich spectra created by the high temperature ICP source. All wavelengths are measured at or very close to the blaze angle to achieve

high dispersion without loss of efficiency. The plasma source is a 27.12 MHz, 2.5 kW tuned oscillator, chosen to accommodate a wide tolerance of sample types. For routine analysis, the spectrometer can determine up to 20 elements in each stored program, with a total capacity of three stored programs.

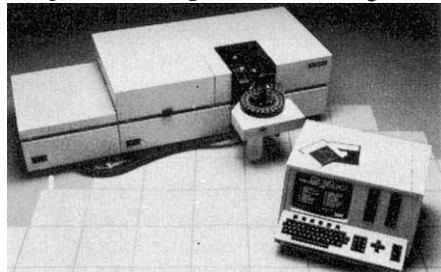
The modular UVIKON EASY-10 **spectrophotometer** by Kontron Instruments combines a double-beam spectrophotometer, a personal computer, EASY-10



*A convenient spectrophotometer for small laboratories.*

software and a four-colour plotter (Reader Service No. 103). The system's data handling capabilities include scanning and time drive for rate assays, and kinetic calculations using linear regression.

Varian has extended its SpectrAA range of atomic absorption spectrometers to include two new instruments equipped with **Zeeman background correction**, for samples exhibiting structured background



*Varian now offers Zeeman background correction in its spectrophotometers.*

signals and spectral interferences (Reader Service No. 104). The SpectrAA 30 and 40 Zeeman spectrophotometers' accuracy of correction is enhanced by using polynomial interpolation of the data points and an extremely rapid duty cycle, to ensure rapid changes in both slope and values of background peaks are accurately monitored. The SpectrAA 40 Zeeman offers an advantage over the SpectrAA



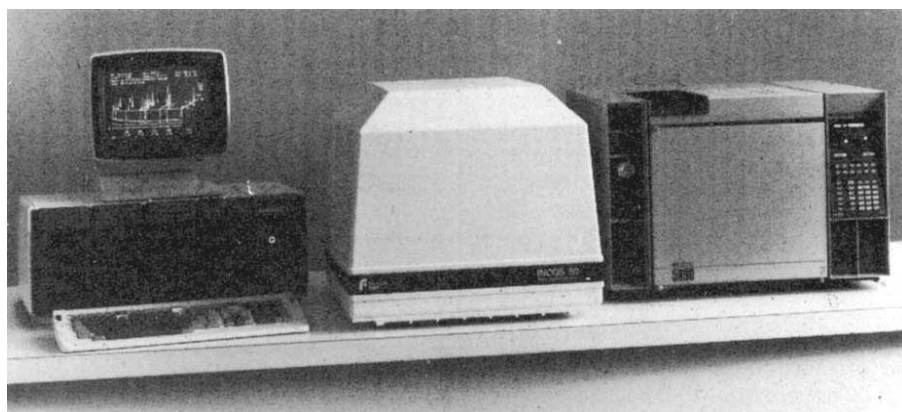
Zeeman 30 because it is designed for unattended multi-element analysis, and automatically selects the appropriate hollow cathode lamp and warms up the next lamp in readiness for the following element in the sequence. Both systems are floppy disk-based, and have unlimited data storage. Up to 12 stored methods can be selected for multi-element analysis, and all instrumental conditions are automatically set as each method is recalled.

**Fluorescence lifetime and transient spectroscopy** is now possible with a commercial instrument (Reader Service No. 105). Edinburgh Instruments' PC-based system combines the functions of a multi-channel analyser with a computer for re-convolution analysis of decay data. The system interfaces with all existing time-correlated single photon counting instruments through a BNC connection to the TAC. Multichannel scaling is included with options to extend up to 8K data channels and external polarizer control.

Short-lived species such as radicals, ions and excited states are not quick enough to elude detection by Applied Photophysics' **laser spectrometer** (Reader Service No. 106). Its latest machine overcomes the problem of producing such species in sufficient concentrations for detection by using a high-powered pulse laser to generate new chemical species in a few nanoseconds. Applied Photophysics claims that chemical reactions that last only one hundredth of a millionth of a second can be studied with the new machine. The computerized data collection facilities of the laser spectrometer allow spectra in the UV and visible region to be captured and complex decay kinetics to be analysed.



Applied Photophysics' laser spectrometer captures data on short-lived species.

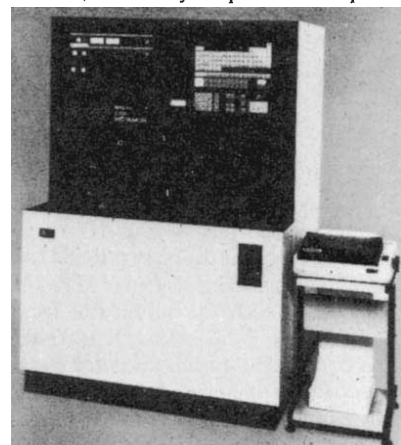


Finnegan MAT's INCOS 50 puts gas chromatography/mass spectroscopy right on the benchtop.

One of Finnegan MAT's newest spectrometers, the INCOS 50, will be making its first US show appearance (Reader Service No. 107). The INCOS 50 is a **benchtop-sized gas chromatography/mass spectrometer** system that Finnegan MAT claims is ideal for laboratories performing environmental monitoring and pre-employment drug screening. Its \$77,000–85,000 (US) price has made it Finnegan MAT's fastest growing product family.

Beckman says that its DU Series 60 **UV-visible spectrophotometers** fit virtually any application in bioresearch and quality control (Reader Service No. 108). Its \$6,700–8,200 (US) scanning spectrometers can provide final values for protein assays, DNA purity and tablet dissolution studies, kinetic and quantitative assays, wavelengths, gel and film scanning, microsampling analysis, and method determination. The \$14,500 (US) DU Series 70 goes one step further by incorporating a CRT display, and by accommodating a range of sampling accessories.

For routine analytical use, Rigaku offers its model 3030 **X-ray spectrometer** (Reader Service No. 109). Rigaku says its \$84,825 (US) system is capable of analysing all elements from fluorine through uranium, and only requires the operator



Rigaku's Model 3030 uses an X-ray.

to press the corresponding element button on the periodic chart panel to initiate the analysis. All analytical parameters and correction factors for groups of up to 15 elements may be preprogrammed, and corrections are automatically made for background, matrix absorption, enhancement, and line overlap. For automatic transmission of analytical data to an external computer, the RS232C interface is available.

Baird & Tatlock Ltd is expanding its range of **UV-visible spectrophotometers** by now distributing Gilford Response spectrophotometers (Reader Service No. 110). The microprocessor-controlled scanning instruments cover an operating range of 200–750 nm, and offer both single and temporal dual-beam readings. The results are displayed on a CRT in both graphic and tabular format, and the operator can choose parameters and manipulate collected data by the same means. The spectrophotometer includes programs of wavelength scanning, kinetics, time scanning, multiwavelength analysis, standard curve plotting and read samples. The photometer calculations available



range from absorbance and first and second derivations to concentration, absorbance ratio, enzyme rate, peak identification, area integration and multiwavelength analysis at five wavelengths. Other models in the series feature automatic positioning of a six-place cuvette holder, twin disk drives for archival data and program storage and additional software for multicomponent analysis and thermal denaturation-renaturation analysis.

The JMS-HX 100, by Jeol, is a **high-performance mass spectrometer** which can be used in MS-MS tandem operations (Reader Service No. 111). The system is also suited for fast atom bombardment ionization techniques, for the measurement of compounds with mass numbers between 3,000 and 6,000, including biological compounds such as glycolipids, proteins and peptides. In its MS-MS mode, the JMS-HX 110 can determine an amino acid sequence of a polypeptide and perform quantitative analysis of trace samples. Masses of 12,500 daltons at a maximum acceleration voltage of 10kV, up to masses of 125,000 daltons at 1kV can be covered. A broad range of ion sources, including EI/CI, EI/CI/DCI, FAB/EI/CI/DCI and FAB/FD combinations are available. According to Jeol, its system has a sensitivity of 0.1 ng of methyl stearate in the EI mode and an ion intensity of  $3 \times 10^{-8}$  coulomb  $\mu\text{g}^{-1}$  with the molecular ion of methyl stearate. The JMS-HX 100's data system corrects all parameters for runs in either positive or negative, controls temperature, and acquires, tabulates, and displays spectra in several ways including three-dimension chromatograms.

Techmation is distributing the Plasmascan 8410 **atomic emission spectrometer** manufactured in Australia by Labtam

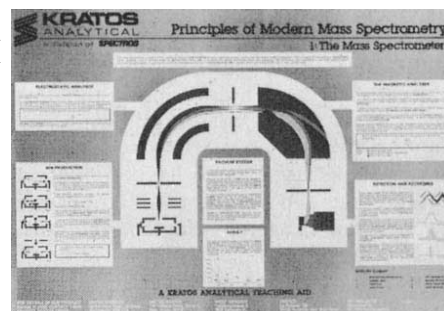
(Reader Service No. 112). Special features of the Plasmascan spectrometer include a demountable torch for ease in cleaning, and a stop/flow GMK nebulizer designed to tolerate many difficult matrices with a high solid content. The alignment of the nebulizer is not critical and its design eliminates particle build-up at the gas/liquid interface. The Plasmascan's integral computer runs Spectrometer Laboratory Information Management Package (Sлимпac) software. A vacuum monochromator with a range of 160 to 820 nm is standard, and systems can be supplied with one or two monochromators fitted as required.

### Literature and more

An application note entitled *Thin Film Analysis XPS Depth Profiling* is available from Kratos Analytical (Reader Service No. 113). The free publication explains **depth profiling by X-ray photoelectron spectroscopy**, a technique finding wide application in quality control laboratories where assessments of surface contamination and thin film analyses are performed.

A 24-page brochure describing SPEX Industries, Inc.'s full line of **light measuring instruments** and accessories is now available (Reader Service No. 114). The brochure includes spectrometers, spectrographs and control systems, and also provides general information to help users decide which type of instrument system best meets their specific analytical needs.

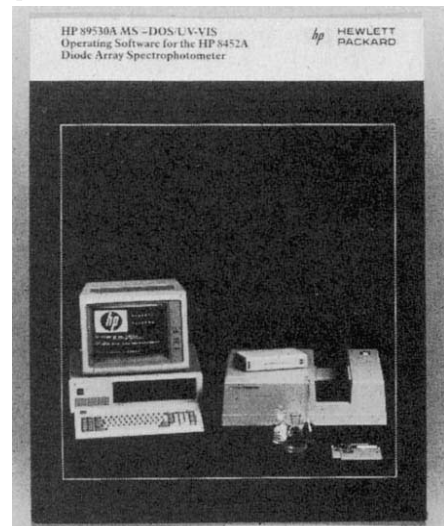
A **full-colour wallchart** available from Kratos Analytical details the essential elements of mass spectrometer design and function (Reader Service No. 115). The chart describes a mass spectrometer with full reference to its constituent parts; ion source, analyser, detector and vacuum



*A good reference for the lab or the classroom.*

system. The free wallchart is intended as both a teaching aid and a memory prompter for laboratory personnel, and also outlines the electrostatic analyser, the magnetic analyser, ion production and detection, and recording.

**Software** made especially for the HP 8452A diode-array UV-visible spectrophotometer is described in a new brochure

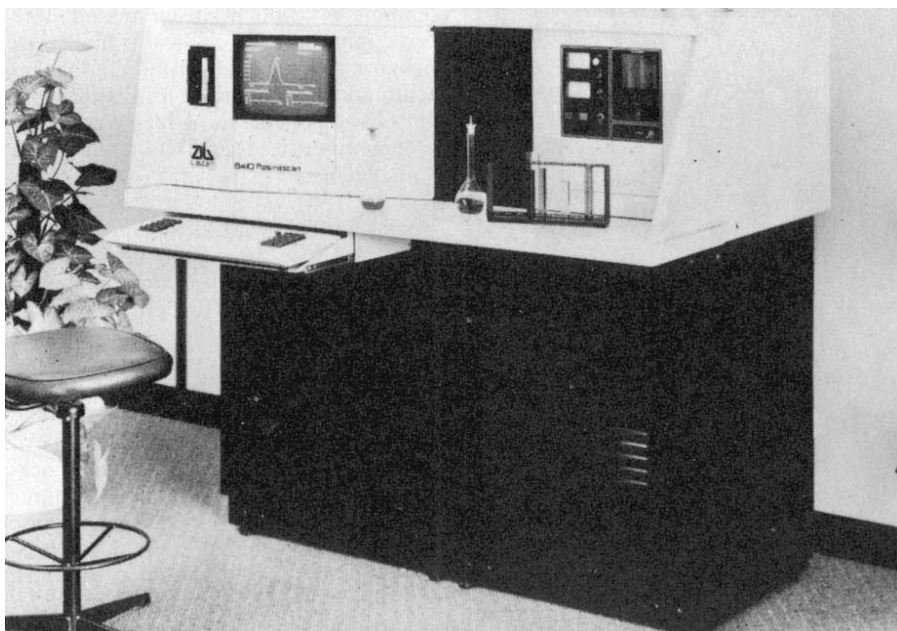


*Describes software for the HP 8452A.*

available from Hewlett-Packard (Reader Service No. 116). The free six-page-booklet outlines the performance features of the software for general scanning, quantitation and kinetics, that runs on most MS-DOS-compatible computers.

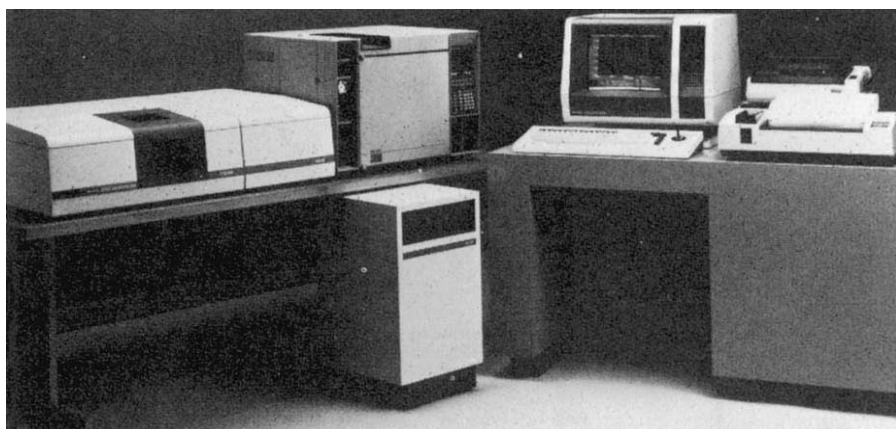
MScan is now offering a **biopolymer structural elucidation service** (Reader Service No. 117). Using high mass fast atom bombardment mass spectrometry, MScan will perform protein and carbohydrate analysis, solving a variety of structural problems including identification of N- and C-terminal blocking groups, checking the integrity of synthetic peptides, and identification of post-translational modifications such as position and type of carbohydrate-protein linkages.

Heyden & Son Ltd has announced the publication of the *Sadtler Infrared Spectra Atlas of Rubber Chemicals* (Reader Service No. 118). A compilation of infrared spectra, physical and chemical data, and supporting information for 589 rubber



*Straight from Australia, the Plasmascan atomic emission spectrometer is loaded with features.*



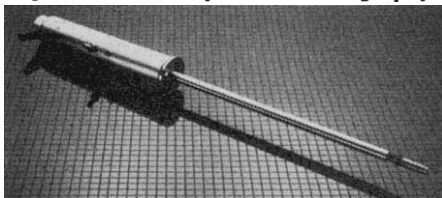


Bio-Rad offers a variety of optical benches that reaches from near- to far-IR.

chemicals grouped by principal function, the **spectra atlas** provides a practical desk reference in a single volume. Spectra are arranged one per page, and FT-IR spectra are presented in a transmittance versus frequency (wave number) format generally over the spectral region 4,000 to 450  $\text{cm}^{-1}$ . Each compound is listed by its commercial or trade name, and accompanying information includes the classification, manufacturer, properties, function or use, and the method used in sample preparation.

### Spectroscopy aids

Kratos Analytical has announced a **continuous flow fast atom bombardment (FAB) probe** to broaden the application of mass spectrometry to allow the analysis of non-volatile compounds such as peptides and organometallics (Reader Service No. 119). The probe contains a fused silica capillary that carries a continuous flow of the sample into the mass spectrometer, enabling FAB to be used for continuous sampling of reactions, and for techniques such as liquid chromatography-



The Kratos Analytical FAB probe is useful for non-volatiles.

mass spectrometry. The probe also increases the sensitivity of FAB mass spectrometry by reducing background peaks. The new probes are now available for the Kratos MS50 and MS890 FAB mass spectrometry units, and existing Kratos instruments can be retro-fit for the new probe, if desired.

Bio-Rad is offering **optical benches** for near-IR, far-IR and vacuum work for the Digilab FTS-40 and FTS-60 spectrometers (Reader Service No. 120). The FTS-40N/FTS-60N near-IR benches feature tungsten halogen sources, quartz beam splitters and PbSe detectors, and have ranges from 400 to 15,000  $\text{cm}^{-1}$ . The far-IR benches are equipped with Hg sources, Mylar

beam splitters and DTGS detectors, giving them a range down to 10  $\text{cm}^{-1}$ , and making them suitable for organometallic, catalyst and zeolite analysis. For superfine work in the far-IR, variously equipped vacuum benches are available. Bio-Rad claims a baseline peak-to-peak signal-to-noise ratio of 1,400:1 for a one second collection time at 1  $\text{cm}^{-1}$  resolution for its benches.

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*These notes are compiled by Carol Ezzell from information provided by the manufacturers. To obtain further details about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.*

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# Occupational trends in the United States

Richard Pearson

*US government figures show just how rapidly employment patterns are changing.*

In September last year the Employment column reported on the occupational changes expected in the United Kingdom over the period to 1990<sup>1</sup>. That study was unique in being based on the expectations of employers and as such it was able to explain many of the underlying causes of change. More frequently, however, economic models or statistical projections are used to map out future occupational change. Their utility is largely dependent on the availability of historical time series of occupational data, complemented by a variety of desk research techniques. The most developed statistical projections of occupational change are those produced biennially by the Department of Labor in the United States. One of the main outlets and uses of the model is in the production of the annual *Occupational Outlook* publication which details occupational and training profiles of over 750 occupations. The underlying model itself draws an extensive set of time series data on 150 in-

expected to grow, albeit at a much slower rate of only about 6 per cent. This growth rate, however, in part represents a recovery after the recession of 1981-82 and in 1995 employment is likely to be only slightly higher than that in 1979. The main engine of this growth is expected to be increasing capital spending and defence expenditure, with the computer, materials handling equipment and scientific and instrument industries particularly benefiting and showing output growth significantly in excess of projected productivity gains. The sectors showing decline include basic industries such as mining, where productivity gains, improvements in technology and in some activities rising import penetration will reduce employment. Agricultural employment is also expected to continue its long-term decline (Fig. 1).

One thing that is not clear from these figures, though, is the extent to which they represent a statistical name change. In the United Kingdom, for example, almost half the growth in service jobs (actual and projected) through the 1980s is expected to be due to the goods producing sectors 'contracting out' activities such as wholesaling, catering, transport and professional services to specialist service sector companies. Thus a crude interpretation of the figures, in the UK case at least, over-emphasizes the so called decline of manufacturing and the growth of services. But it is not clear whether the United States is following a similar trend.

A more balanced perspective can be had from an assessment of occupational change, although these figures are in part derived from industrial or sectoral trends. The occupations with the highest growth rates will be paralegal, computing and electronics staff, and medical assistants all showing increases of 50 per cent or more in employment levels. In absolute terms, then cashiers, nurses, cleaners, truck drivers and waiters are all groups expecting to expand their employment by 400,000 jobs or more (Table 1). A detailed analysis shows no general upgrading of jobs to graduate status, there being no more growth

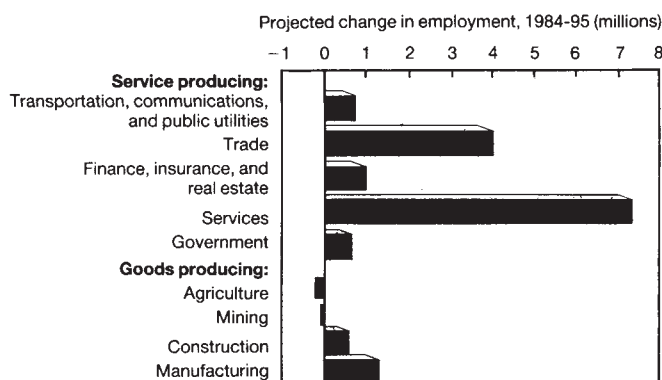


Fig. 1 Data from the US Department of Labor study show that some industries will grow much faster than others during the 1990s.

among jobs requiring extensive training than those requiring little formal training, and only one in four of the new jobs is expected to require a college degree. The occupations showing the largest decline are stenographers (down 40 per cent), shoe machinists (down 32 per cent), and railroad operators (down 26 per cent), these are to be found either in the declining sectors or are being severely affected by technological change, the stenographers being a prime example.

For scientists and engineers the best prospects are for those related to computing and electronics, and engineers in general are expected to have better job prospects than scientists although both groups are expected to show at least average employment growth. Military technology, the automation of industrial production, the development of energy sources, basic research, and environmental protection are all activities generating improved employment prospects for such staff.

Overall the rate of population growth in the United States is slowing down and the total labour force is expected to grow a little slower than will employment opportunities. The final balance in 1995 will be subject to the many uncertainties in the economy, as well as the rate of technological change, and changing social habits and priorities. Nevertheless, the outlook in terms of job opportunities is expected to continue to improve, and will encompass a wide range of skill levels and locations. Flexibility will be the key need for job seekers operating in such a rapidly changing market. □

1. Pearson, R. *Nature* 323, 94 (1986).

2. *Occupational Projections and Training Data* (US Department of Labor, 1986).

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Table 1 Occupations with the largest job growth, 1984-95 (numbers in thousands)

| Occupation                               | Change in employment 1984-95 |          |
|------------------------------------------|------------------------------|----------|
|                                          | Number                       | Per cent |
| Cashiers                                 | 556                          | 29.8     |
| Registered nurses                        | 452                          | 32.8     |
| Janitors and cleaners                    | 443                          | 15.1     |
| Truckdrivers                             | 428                          | 17.2     |
| Waiters and waitresses                   | 424                          | 26.1     |
| Wholesale trade                          | 369                          | 29.6     |
| Nursing aides, orderlies, and attendants | 348                          | 28.9     |
| Salespersons, retail                     | 343                          | 12.6     |
| Accountants and auditors                 | 307                          | 34.8     |

Data from ref. 2.

dustries and 750 occupations making it the most sophisticated analytical econometric occupational model in the world.

From a base of 107 million in 1984, total employment in the United States is expected to grow to 130 million in 1995, a growth of about 15 per cent. This represents a slowdown in the growth of the previous decade. Service industries which account for 7 out of 10 jobs are expected to continue to grow more rapidly than manufacturing and other industries. This growth will be fuelled by rising living standards, in turn leading to greater demand for health care, entertainment, banking and insurance services, and these sectors are expected to account for nine out of ten new jobs over this period. Employment in US goods producing sectors is also